

Remarks

In view of the above amendments and the following remarks, reconsideration of the outstanding office action is respectfully requested.

Claims 38-55 have been cancelled without prejudice, and new claims 106-108 have been introduced. Descriptive support for new claims 106 and 107 is provided in the paragraph at page 15, lines 16-27. Descriptive support for new claim 108 is provided at page 20, lines 10-16. Claims 17-37, 56-65, and 106-108 remain pending and under examination. No excess claim fees are due with this submission.

This submission is accompanied by a request for a three-month extension of time (from the deadline of August 28, 2008) and an Information Disclosure Statement (IDS). Fees for the extension of time and IDS have been withdrawn from deposit account 14-1138. Any excess fees can be credited and any fee deficiencies can be charge to this same account.

The rejection of claims 27-37 under 35 U.S.C. § 112, first paragraph, as being enabling only for the treatment of a thrombotic condition, but not for the prevention of a thrombotic condition is respectfully traversed. The data presented in Examples 6 and 8 fully supports the prophylactic method of use as claimed. Because the data demonstrates that platelet activity can be blunted (by pretreatment with a PPAR γ agonist), this fully supports the prevention of unwanted platelet activation and, hence, unwanted thrombosis. Therefore, the rejection of claims 27-37 for lack of enablement is improper and should be withdrawn.

The rejection of claims 23-25, 33-35, and 62-65 under 35 U.S.C. § 112, second paragraph, for indefiniteness is respectfully traversed. Claims 23, 33, and 62 recite use of “*an* inducer of a PPAR γ agonist” whereas claims 24, 34, and 63 recite that “*the* inducer of a PPAR γ agonist” is a particular agent. Thus, claims 23, 33, and 62 provide proper antecedent basis for this language. Moreover, the use of several inducers of a PPAR γ agonist are described and claimed in the present application. Therefore, this rejection is improper and should be withdrawn.

The rejection of claims 17-23, 25, 27-33, 35, 37, 56-62, and 64 under 35 U.S.C. § 103(a) for obviousness over the combination of U.S. Patent No. 6,127,394 to Pershadsingh et al. (“Pershadsingh”) in view of U.S. Patent No. 7,018,985 to Boyer et al. (“Boyer”) is respectfully traversed.

Pershad Singh identifies a class of thiazolidinedione derivatives and indicates that the compounds are activators of PPAR γ (col. 11, line 13), although other portions of the reference are unclear whether these compounds are agonists or antagonists of PPAR γ (col. 11, lines 13-14). Among a remarkably long list of disparate indications (over 200 diseases or conditions listed in Tables II-VII), Pershad Singh identifies “thrombosis and restenosis after angioplasty” and “myocardial infarction” in Table II. There is, however, no experimental evidence cited in Pershad Singh to support either of these uses, nor is there any reference to prior art support for these therapeutic uses with related compounds (i.e., other thiazolidinediones).

Boyer is cited primarily as background evidence, which the PTO relies on to demonstrate that platelet adhesion and activation were known to be critical events in intravascular thrombosis.

Based on the combination of Pershad Singh and Boyer, the PTO asserts at pages 8-9 of the office action that the treatment of thrombosis as described by Pershad Singh would have involved prevention of platelet activation and aggregation, as taught by Boyer. Applicants respectfully disagree.

Prior to the present invention, it was not known that platelets possessed the nuclear receptor PPAR γ . Quite the contrary, persons of skill in the art would have expected that platelets did not possess PPAR γ , because platelets lack a nucleus. Given this expectation, persons of skill in the art would have had no basis whatsoever to expect that contacting a platelet with a PPAR γ agonist would have any effect at all. Indeed, the present invention involved the identification of a surprising, new non-transcriptional function for PPAR γ . The present application demonstrates for the first time that contacting a platelet with a PPAR γ agonist can inhibit platelet release of CD40 ligand (as well as thromboxanes and prostaglandins), and also inhibit expression of CD40 ligand on the platelet surface. None of these changes in platelet activity would have been expected by persons of skill in the art given the prior expectation that platelets do not possess the nuclear receptor PPAR γ .

The PTO acknowledges at page 8 that Pershad Singh fails even to mention platelets. In fact, the word ‘platelet’ only appears in the specification of Pershad Singh in the context of conducting blood work to assess a hypothetical patient profile, e.g., “[a]dditionally, a complete blood count, including white cell count and differential, platelet count, and liver function tests...are checked prior to treatment and periodically thereafter” (Example 4, col. 21, lines 4-8). This is mentioned only in the *prospective* examples of

Pershadsingh. And, as noted above, the term “thrombosis” only appears among a rather lengthy list of disparate indications.

Given this expectation and the absence of any teaching whatsoever in Pershadsingh concerning the ability of PPAR γ agonist to blunt the activity of platelets, persons of skill in the art would not have expected the contacting of platelets with a PPAR γ agonist to be useful in preventing thrombus formation, treating/preventing a thrombotic condition or disease, or preventing platelet aggregation as presently claimed. That platelet activation is involved in thrombus formation—the reason for the PTO’s reliance on Boyer—is beside the point. There simply was no expectation in the prior art that the claimed invention could be achieved by contacting platelets with a PPAR γ agonist.

Moreover, as further evidence of the nonobviousness of the presently claimed invention, applicants submit herewith two post-filing date references that acknowledge the contribution of the present invention. The underlying research of the present application was published in the research journal *Blood* in 2004 (Akbiyik et al., “Human Bone Marrow Megakaryocytes and Platelets Express PPAR γ , and PPAR γ Agonists Blunt Platelet Release of CD40 Ligand and Thromboxanes,” *Blood* 104:1361-1368 (2004) (“Akbiyik”). The Akbiyik reference has been cited numerous times (at least 24) by non-inventors, including in reviews by Santilli et al., “CD40/CD40L System and Vascular Disease,” *Intern Emerg Med.* 2:256-268 (2007) (“Santilli,” copy attached as Exhibit 1) and Borchert et al., “Review of Pleiotropic Effects of Peroxisome Proliferator-Activated Receptor γ Agonists on Platelet Function,” *Diabetes Technology & Therapeutics* 9(5):410-420 (2007) (“Borchert,” copy attached as Exhibit 2). Santilli recites, at page 262, that the work of the present inventors “identify[ies] in the platelet a *new* target cell for [thiazolidinediones]” (emphasis introduced). Borchert credits the present inventors, with citation to the Akbiyik reference at page 413, as the first to discover that platelets contain large amounts of PPAR γ protein.

Because the discovery underlying the present invention represents a paradigm shift in the understanding of platelet function, persons of skill in the art would have recognized at the time the present invention was made—and, indeed, have since—that the platelet represents a new target for PPAR γ agonists. As such, persons of ordinary skill in the art would not have expected before October 2003 that contacting mammalian platelets with a PPAR γ agonist would “inhibit[] formation of a thrombosis by the mammalian platelets” (claim 17), “inhibit[] platelet activation to treat or prevent the thrombotic condition or disorder” (claim 27), or “inhibit[] aggregation of the mammalian platelets” (claim 56).

For these reasons, the obviousness rejection of claims 17-23, 25, 27-33, 35, 37, 56-62, and 64 over the combination of Pershadsingh in view of Boyer is improper and should be withdrawn.

The rejection of claims 24, 26, 34, 36, 63, and 65 under 35 U.S.C. § 103(a) for obviousness over the combination of Pershadsingh and Boyer, as cited above, further in view of U.S. Patent No. 6,413,931 to Höök et al. (“Höök”) is respectfully traversed.

The teachings and deficiencies of the combination of Pershadsingh and Boyer are noted above. Höök describes the use of decorin to bind fibrinogen in the presence of zinc (Zn^{2+}) to prevent fibrin clot formation. The PTO has asserted that one of skill in the art would have used decorin to treat thrombosis given the teachings of Höök. Applicants respectfully disagree for several reasons.

Firstly, Höök fails to overcome the above-noted deficiency of the combination of Pershadsingh and Boyer.

Secondly, the claim language requires “administering ... an inducer of a PPAR γ agonist to a mammal in a manner that *provides for said contacting*” (claims 23 and 62) or “administering ... an inducer of a PPAR γ agonist to the individual in a manner that *provides for said contacting*” (claim 33), or administering a DNA molecule encoding the inducer, which also is “*effective to cause said contacting*” (as recited in claims 26, 36, and 65). Thus, the use of an inducer of a PPAR γ agonist, such as decorin, or a DNA molecule encoding the same, is not for directly acting on fibrinogen as described by Höök, but instead for the entirely different purpose of inducing production of a native PPAR γ agonist, which in turn contacts the platelets. Because Höök fails to teach or suggest this new use of decorin or a DNA molecule encoding decorin, the combination of Pershadsingh, Boyer, and Höök is deficient in this regard.

For these reasons, the rejection of claims 24, 26, 34, 36, 63, and 65 for obviousness over the combination of Pershadsingh, Boyer, and Höök is improper and should be withdrawn.

Applicants further submit that new dependent claims 106-108 are patentable for all of the reasons noted above. Moreover, with respect to new claim 108, applicants note that the teachings of Pershadsingh regarding thrombosis concern “thrombosis and restenosis after angioplasty.” Because angioplasty is known to cause mechanical trauma to the endothelium, thrombosis and restenosis often occurs; however, this type of mechanical

induction of thrombosis and restenosis is very different from the aberrant thrombosis that is caused by a disease state (*i.e.*, in the absence of mechanical trauma). Therefore, Pershadsingh is also deficient with regard to new dependent claim 108.

In view of all of the foregoing, applicants submit that this case is in condition for allowance and such allowance is earnestly solicited.

Respectfully submitted,

Date: November 26, 2008

/Edwin V. Merkel/
Edwin V. Merkel
Registration No. 40,087

Nixon Peabody LLP
1100 Clinton Square
Rochester, New York 14604
Telephone: (585) 263-1128
Facsimile: (585) 263-1600

Exhibit 1: Santilli et al., “CD40/CD40L System and Vascular Disease,”
***Intern Emerg Med.* 2:256-268 (2007)**

F. Santilli • S. Basili • P. Ferroni • G. Davì

CD40/CD40L system and vascular disease

Received: 16 February 2007 / Accepted in revised form: 28 May 2007 / Published online: 23 November 2007

Abstract Several distinct lines of investigation in the context of atherosclerosis dealing with low-grade inflammation, oxidative stress and platelet activation are now emerging, with CD40/CD40L system as the missing link. CD40 ligand is a transmembrane glycoprotein structurally related to tumour necrosis factor- α and more than 95% of the circulating CD40L derives from platelets. CD40L appears as a multiplayer of several cell types in the inflammatory network. The peculiarity of CD40L as an inflammatory mediator derived from platelets expands the functional repertoire of platelets from players of haemostasis and thrombosis to powerful amplifiers of inflammation by promoting the release of cytokines and chemokines, cell activation and cell–cell interactions. The multifunctional role of CD40L, as a simultaneous activator of all these systems, further blurs the intricate relationship between such events both in the physiological systems and the pathological derangement occurring in atherothrombosis.

Keywords sCD40L • Inflammation • Atherosclerosis • Thrombosis

F. Santilli • G. Davì (✉)
Center of Excellence on Aging
University of Chieti “G. D’Annunzio” School of Medicine
Via Colle dell’Ara, I-66013 Chieti, Italy
e-mail: gdavi@unich.it

S. Basili
Department of Medical Therapy
University of Rome “La Sapienza”
Rome, Italy

P. Ferroni
Department of Laboratory Medicine & Advanced Biotechnologies
IRCCS San Raffaele, Rome, Italy

Introduction

CD40L is gaining much attention for its role in the initiation and progression of atherosclerosis. Several distinct lines of investigation in the context of atherosclerosis dealing with low-grade inflammation, oxidative stress and platelet activation are now emerging, with CD40/CD40L system as the missing link. CD40 ligand (CD40L, CD154) is a 39-kD transmembrane glycoprotein structurally related to tumour necrosis factor- α (TNF- α), originally thought to be restricted to stimulated CD4-positive T cells [1, 2], mast cells and basophils [3]. Subsequent studies demonstrated its expression on platelets, carrying preformed CD40L, which is rapidly translocated onto the cell surface following activation [4]. The receptor for CD40L, CD40, is constitutively expressed on monocyte/macrophages, endothelial cells (ECs), smooth muscle cells (SMCs) [5] and platelets [6]. Studies on the cellular distribution of CD40L indicate that more than 95% of the circulating CD40L derives from platelets [7]. Henn and colleagues have shown that CD40L, cryptic in unstimulated platelets, is expressed on the surface of platelets within seconds of platelet activation and then cleaved to generate a soluble, trimeric fragment, sCD40L [4]. Multiple platelet agonists, including collagen, thrombin and ADP, are able to induce the exposure of sCD40L from platelets [4]. The translocation of sCD40L on the platelet surface requires a few seconds or minutes, similar to the expression of P-selectin, whereas the shedding of the molecule into the circulation is a slower event, lasting 30–45 min depending on the agonist inducing activation [8]. It is now generally accepted that sCD40L is generated by proteolytic cleavage mediated through activation of a membrane-bound protease, possibly a metalloproteinase (MMP), although the involvement of intracellular structures and reactions has been also proposed, i.e., actin polymerisation [9, 10]. In addition, sCD40L may be possibly involved in a self-perpetuating feedback loop, whereby it binds platelet-bound

CD40 leading to further proteolysis of membrane-bound CD40L, with consequent generation of further sCD40L [6].

There are conflicting data as to whether sCD40L stimulates resting platelets by binding to constitutively expressed CD40 during direct cell-cell contact, thus eliciting proinflammatory responses. Inwald et al. demonstrated granule release and enhanced P-selectin expression after incubation of platelets with trimeric sCD40L [11], suggesting that the biological activity of sCD40L may depend on its existence in a biologically active trimeric structure (as in the case of membrane-bound CD40L) [12]. The prothrombotic activity of sCD40L may be attributable to its KGD peptide sequence as infusion of sCD40L with an altered KGD sequence did not reverse the normal phenotype in CD40L-deficient mice [7]. This sequence in turn allows its binding to glycoprotein IIb/IIIa, with consequent stabilisation of arterial thrombi [7]. Proof that sCD40L is a GPIIb/IIIa ligand was obtained through experiments of direct binding of sCD40L to purified glycoprotein IIb/IIIa [7]. More recently, *in vitro* studies showed that GPIIb/IIIa antagonists (eptifibatide, abciximab and tirofiban) are capable of inhibiting the release of sCD40L in a dose-dependent manner [8, 10, 13], although translocation of CD40L from intraplatelet stores to the surface was unmodified [10]. Taken together, these observations strongly implicate CD40L in the triggering and perpetuation of platelet activation.

CD40/CD40L interactions have also been involved in inflammation and thrombosis. In fact, CD40 and CD40L are coexpressed by virtually all of the cells involved in the processes of atherosclerosis at all stages, such as vascular endothelial cells, smooth muscle cells, macrophages, activated T lymphocytes and platelets [14]. CD40/CD40L interaction on these cellular types triggers a series of events occurring in the vascular wall and in the circulation during the ongoing inflammatory response, events which taken together identify the inflammatory and prothrombotic phenotype observed in both the early and late stages of atherosclerosis. Ligation of CD40 on ECS and VSMCs induces the expression of adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1), intercellular cell adhesion molecule-1 (ICAM-1) and P-selectin, which in turn promote the recruitment and extravasation of monocytes and lymphocytes at the site of vascular injury [14]. Further recruitment of lymphocytes is elicited by CD40L-induced secretion of cytokines such as interleukin-1 (IL-1), interleukin-6 (IL-6) and tumour necrosis factor- α (TNF- α) by several cells, thus fostering a predominantly Th1 cytokine-driven immune reaction, characteristic for atherogenesis, and of chemokines, such as macrophage inflammatory protein-1 α (MIP-1 α), MIP-1 β regulated upon activation normal T-cell expressed and secreted (RANTES), stromal derived factor-1 (SDF-1) and monocyte chemoattractant protein-1 (MCP-1) [5]. CD40/CD40L signalling in endothelial cells results in the production of reactive oxygen species (ROS), which antagonise endothelial NO production thus promoting endothelial dysfunction. In addition, this signalling also results in upregulation of the interstitial collagenases MMP-

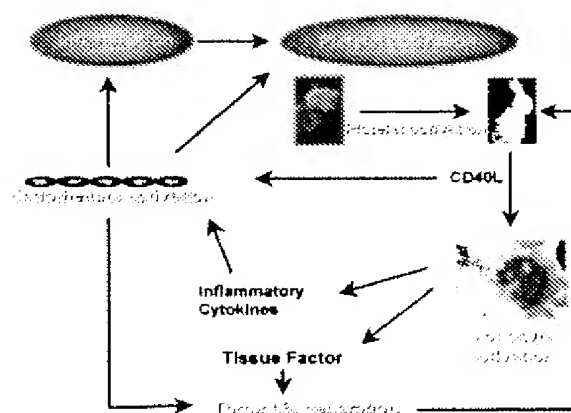


Fig. 1 Role of CD40L in the complex interplay between inflammation, endothelial activation/dysfunction and platelet/coagulative activation. Soluble CD40L, shed from platelets upon platelet activation, triggers monocyte activation, leading to release of inflammatory cytokines and TF expression with thrombin generation, and activation of the endothelium, resulting in further inflammation and platelet activation

1, MMP-8 and MMP-13, contributing to impairment of plaque stability, and in inhibition of endothelial cell migration, thus preventing reendothelialisation of plaque erosion. Finally, CD40 signalling induces tissue factor (TF) expression [15–17], which, while promoting blood coagulation, is also able to activate platelets, which in turn enhance further CD40L shedding with consequent amplification of the inflammatory reaction. In addition, sCD40L increases stimulation-induced platelet release of ROS through activation of Akt and p38 MAP kinase signalling pathways [18]. Recent *in vitro* and *in vivo* experiments in a mouse model elucidated a novel alternative pathway for CD40L-mediated inflammation, by interaction with the monocyte/macrophage integrin MAC-1. This interaction enhances adhesion and migration of inflammatory cells and myeloperoxidase release *in vitro*, and inhibition of MAC-1 in low-density lipoprotein (LDL) receptor-deficient mice attenuates lesion development and decreases macrophages accumulation [19].

All these events may potentially induce or facilitate an acute thrombotic event (Fig. 1).

CD40L and vascular disease

Coronary artery disease

The pivotal role of platelet activation in atherothrombosis, coupled with the finding that most of sCD40L is derived from platelets, has made CD40L an interesting subject in the setting of cardiovascular disease and of acute coronary syndrome (ACS).

Clinically relevant concentrations of human sCD40L increased the expression of its receptor CD40 in human coronary artery endothelial cells through a mechanism mediated by oxidative stress and extracellular signal-regulated kinase (ERK) 1/2 activation, suggesting a mechanism of amplification of CD40L biological function contributing to endothelial dysfunction and platelet activation in the coronary endothelium [20].

Elevated levels of sCD40L have been reported in patients with both stable or unstable angina (UA) [21], acute myocardial infarction (AMI) [22] and diabetes with angiographically proven coronary artery disease (CAD) [23]. Aukrust and colleagues also performed a series of experiments *ex vivo* to test the hypothesis that the CD40/CD40L dyad may play a pathogenetic role in both the long-term atherosclerotic process and in the triggering of ACS [21]. They also addressed the important issue of the cellular origin of the enhanced sCD40L. In fact, they showed that platelets are able to release large amounts of sCD40L *ex vivo* when stimulated with the thrombin receptor-antagonist peptide SFLLRN in patients and controls. Moreover, increased surface expression of CD40L on T lymphocytes and increased *in vitro* shedding of sCD40L from these cells has also been reported in patients with UA [21]. Thus, T lymphocytes and particularly platelets cause the major contributor to increased circulating sCD40L in the setting of UA. Interestingly, sCD40L-rich serum from patients with UA was able to induce enhanced release of MCP-1 from monocytes, giving rise to the hypothesis that sCD40L may be a contributor rather than the consequence of UA, being responsible for the progressive plaque instability and eventually plaque rupture. On the other hand, procedures such as cardiopulmonary by-pass [24] and percutaneous transluminal coronary angioplasty (PTCA) [25] have been shown to induce sCD40L release, suggesting that elevated sCD40L could be a mere consequence of plaque rupture. To recompose these apparently opposite findings in a unifying hypothesis, it has been speculated that repeated episodes of "minor plaque ruptures" may occur before the onset of clinically manifest coronary event, inducing further elevation of sCD40L, and such a vicious circle may be operating in these patients and may contribute to the progression of the disease.

Some additional information about this issue emerges from the study by Cipollone and coworkers [25] and subsequent studies [26], which demonstrated that preprocedural levels of sCD40L are predictive of enhanced inflammatory response and restenosis after PTCA. A mechanistic basis for this clinical study is provided by the work by Urbich et al., who showed that CD40L inhibits endothelial cell migration by increasing production of ROS [27], thus providing a mechanism by which sCD40L generated by thrombosis could promote restenosis.

A prospective study demonstrated that raised sCD40L is a risk factor for future cardiovascular events in healthy

women [5]. Heeschen and coworkers further established the predictive value of this marker by evaluating the clinical relevance of sCD40L in patients presenting with chest pain [22]. In 1088 patients from the c7E3 Fab Antiplatelet Therapy in Unstable Refractory Angina (CAPTURE) Study group, increased sCD40L among patients with chest pain undergoing PTCA identified subjects at risk of death and non-fatal AMI. The increased risk associated with elevated sCD40L was reduced with abciximab pretreatment, a potent platelet glycoprotein IIb/IIIa receptor antagonist. In contrast to troponins, the predictive value of sCD40L levels with respect to the benefit of abciximab is independent of the presence of recent AMI. As a matter of fact, sCD40L identifies the subgroup of patients at highest risk for cardiac events even among those negative for troponin. Whereas positivity of troponins in patients with ACS indicates myocardial necrosis, sCD40L levels reflect the inflammatory thrombotic activity of the culprit lesion in recruiting activating platelets. As the outcome of ACS is highly dependent on the interplay between inflammation and thrombosis, an approach including measurements of these two processes in addition to markers of necrosis may improve our understanding of the underlying pathophysiological processes and allow better assessment of plaque instability.

A subsequent study [28] evaluating plasma sCD40L levels in patients with ACS who eventually reached the study endpoint (death/AMI/congestive heart failure) and in patients who did not, showed significantly higher levels of sCD40L in cases than controls. Patients with the highest sCD40L levels were significantly more likely to develop an AMI or the composite endpoint of death/AMI compared to the patients in the lowest quartiles. Hazard ratios adjusted for troponin I and CRP showed that sCD40L is an independent predictor of the risk of death or MI at 10 months after an ACS.

Recently, genetic polymorphism C807T of platelet glycoprotein Ia has been shown to increase the risk for premature MI and is an independent predictor of sCD40L levels during the acute phase of premature MI and one year after the event [29].

Moreover, in patients with non-ST elevation MI a single nucleotide polymorphism (-3459 A>G) in the CD40L gene is a novel regulator of sCD40L concentrations, and increased sCD40L are predictive of MI and of the efficacy of antithrombotic treatment with dalteparin [30].

These studies do not have the power to establish the causal role of CD40L in the pathogenesis of ACS, but these findings raise the question whether elevated sCD40L levels are a reflection of the pathogenetic role of CD40L in the inflammatory and thrombotic processes or whether they are a mere consequence of the clinical event, namely the result of platelet release after thrombus formation. Even in the latter case of sCD40L elevation as an epiphenomenon, it may constitute a reliable marker of platelet activation and of unstable, prone to rupture plaque. Given

the above findings, it would be worthwhile to evaluate whether lowering the levels of this molecule translates into an improvement of cardiovascular outcomes.

Cerebrovascular disease

Elevated plasma levels of sCD40L were detected in patients undergoing high-resolution magnetic resonance imaging of carotid atheroma with evidence of intraplaque lipid pool [31].

Our group has recently evaluated 42 patients with asymptomatic low-grade carotid stenosis and at least one cardiovascular risk factor [32]. Patients had higher sCD40L as well as C reactive protein (CRP) and IL-6 than controls. Subjects were reviewed annually for a median follow-up of 8 years and 14 patients experienced a cardiovascular event. Cox regression analysis showed that only high sCD40L plasma levels predicted cardiovascular risk, independently of cardiovascular risk factors. Thus, downregulation of this system may represent a potential therapeutic target capable of inducing a more stable carotid plaque phenotype.

Both the ligand and its receptor are overexpressed in human and experimental atherosclerotic plaques, in particular in rupture-prone or ruptured plaques [14].

The CD40L expression on platelets from patients with ischaemic stroke was higher than on platelets from patients with asymptomatic carotid stenosis and from normal subjects [33]. Garlachs and colleagues [34] show CD40/CD40L upregulation in patients with acute cerebral ischaemia. Patients exhibited a significant increase of platelet and T-cell expression of CD40L, as well as of CD40 on monocytes, as compared to controls. Interestingly, CD40L upregulation persisted after 3 months since the initial event. In this light it would be interesting to examine whether sCD40L in the subacute phase may predict the recurrence of ischaemic events [35].

Recently, enhanced sCD40L levels were described in patients with Alzheimer's disease, suggesting a role for CD40L in the pathogenesis of this disease [36]. It is possible to hypothesise that increasing CD40L is a surrogate indicator for the switch from a more stable collagen-rich phenotype to an unstable, rupture-prone phenotype [37], which is ultimately responsible for an acute cerebral ischaemic event or for its recurrence. Whether inhibition of CD40/CD40L dyad may translate into a reversal of such a phenotypic switch remains to be elucidated through intervention studies.

Peripheral artery disease

Recently, the hypothesis of a role for sCD40L in the setting of peripheral artery disease was tested both cross-sectionally and with an intervention study [38]. Plasma sCD40L,

in addition to other platelet indices such as soluble P selectin, platelet microparticles and platelet surface expression of CD62 and CD63, is raised in peripheral atherosclerosis and is increased after peripheral artery angioplasty, although levels seem unrelated to clinical severity. sCD40L levels did not correlate with other markers, suggesting that platelets may not be the unique source of sCD40L, and that other cells may contribute to plasma levels.

Pulmonary arterial hypertension

Pulmonary arterial hypertension (PAH) is a complex disease that recognises both inflammation and platelet activation in its pathophysiology [39]. Damas and coworkers have recently reported an upregulation of the CD40/CD40L system even in this setting [40]. Patients with primary and secondary PAH but not those with chronic thromboembolic pulmonary hypertension had increased plasma sCD40L compared to controls. In addition, in the patient group as a whole, a positive correlation was found between sCD40L and prothrombin F1+2, suggesting CD40L and platelet activation associated with the ongoing thrombus formation in this setting. Prostacyclin therapy for 3 months did not affect sCD40L levels, despite clinical benefit. This study suggests that CD40L may play a role in the pathogenesis of PAH, possibly triggering a complex chain of events played by platelets and ECs through chemokine-related mechanisms.

Heart failure

Stumpf et al. [41] have recently shown enhanced expression of CD40L on the platelet surface during chronic heart failure (HF), further supporting enhanced platelet activation in this disorder.

Serum levels of sCD40L were measured in 236 patients with acute HF following MI treated with either angiotensin-converting enzyme (ACE) inhibition or angiotensin II blockade and followed for 2 years, and in 116 patients with chronic HF [42]. Patients with acute HF had increased sCD40L levels, particularly those with severe HF, diabetes or hypertension; when these patients were followed longitudinally, persistently raised sCD40L levels were found throughout the observation period with no effect of captopril or losartan; the increase in sCD40L during follow-up was not seen in patients receiving warfarin therapy; patients with chronic HF also had raised sCD40L, significantly correlated with clinical severity, neurohormonal dysregulation and left ventricular dysfunction; studies from different blood compartments suggest that the vasculature of lower extremities and the failing myocardium itself may produce and secrete sCD40L in chronic HF.

The correlation between sCD40L and LV dysfunction suggests that increased sCD40L levels in HF may not only be a parameter of platelet activation but could also reflect other pathogenic mechanisms in myocardial failure, including activation of MMPs and induction of inflammatory cytokines and chemokines. Whatever the mechanisms, the CD40-expressing cardiomyocytes may directly interact with CD40L, either in its soluble form or expressed on the surface of infiltrating T cells and platelets, contributing to persistent tissue inflammation and remodelling within the failing myocardium. These findings suggesting enhanced release of sCD40L within the coronary circulation further support enhanced CD40/CD40L interaction within the myocardium.

CD40L and cardiovascular risk factors

Diabetes mellitus

The strong correlation between diabetes mellitus (DM) and atherosclerosis suggests that both conditions may share a common background [43]. A clustering of variables related to chronic low-grade inflammation, oxidative stress and platelet activation is emerging as a conceivable "common soil" that may influence the development of both diseases [44].

In vivo platelet activation has been reported previously in diabetes [45, 46] and increased plasma levels of CD40L have been described recently in both type 1 (T1) and type 2 (T2) DM [23, 47]. Moreover, significantly increased co-expression of CD40 and CD40L on platelets of diabetic patients compared with nondiabetic controls has been reported, with a significant correlation of sCD40L with CD40L expression on platelets [48]. Surface expression of collagen receptor Fc receptor-gamma/glycoprotein VI is enhanced on platelets in T2DM and mediates release of CD40L and activation of endothelial cells, thus suggesting enhanced collagen-mediated platelet activation in diabetes contributing to thrombohaemic complications [49].

In T1DM, enhanced sCD40L levels have been detected in patients both with [50] or without micro- and macrovascular complications [51]. Serum CD40L is an independent determinant of intima-media thickness of carotid artery in young Japanese T1DM patients [52]. Patients with T1DM without other cardiovascular risk factors show increased CD40L expression and platelet-monocyte aggregation [53], possibly contributing to the prothrombotic and proinflammatory milieu found in diabetes. In addition, increased sCD40L levels in patients with T1DM and microangiopathy have been associated with platelet hyperactivity, as assessed by platelet P-selectin expression and soluble P-selectin [50].

The highly significant correlation between plasma CD40L levels and the urinary excretion rate of 11-dehydro-TXB₂, a non-invasive index of *in vivo* platelet activation,

supports the likelihood of CD40L release during TXA₂-dependent platelet activation in T2DM [54]. This relation is in keeping with previously reported evidence that CD40L is rapidly upregulated during platelet activation [4] and that platelet CD40 itself provides a mechanism for platelet activation [11]. Diabetics showed significantly reduced plasma CD40L after 7 days of aspirin. In addition, improved metabolic control after a 4-week intensive diabetes programme led to reduction in both sCD40L and 11-dehydro-TXB₂ [54]. The observation that both improved metabolic control and low-dose aspirin, two independent interventions down-regulating platelet activation in this setting, significantly reduced plasma CD40L levels, without any measurable impact on systemic inflammation, as reflected by the non-significant change in CRP plasma levels, strengthens the hypothesis of a contribution of platelets to enhanced CD40L release in T2DM [54]. In addition, the positive correlation observed between enhanced sCD40L and 8-isoPGF_{2α} is in line with the report of increased production of endothelial ROS by CD40L [27], suggesting that in T2DM the release of sCD40L from activated platelets may contribute to increased oxidant stress. Increased lipid peroxidation and persistent platelet activation have previously been reported in patients with T2DM [45, 46]. Thus, on the basis of these findings, a possible vicious cycle may be suggested in which inflammatory stimuli involving CD40L upregulation induce increased lipid peroxidation with consequent platelet activation resulting in further oxidant stress [4] (Fig. 2).

Recently, Lim et al. reported a strong correlation between sCD40L and both IL-6 and TF, supporting a link between the CD40/CD40L system and hypercoagulable

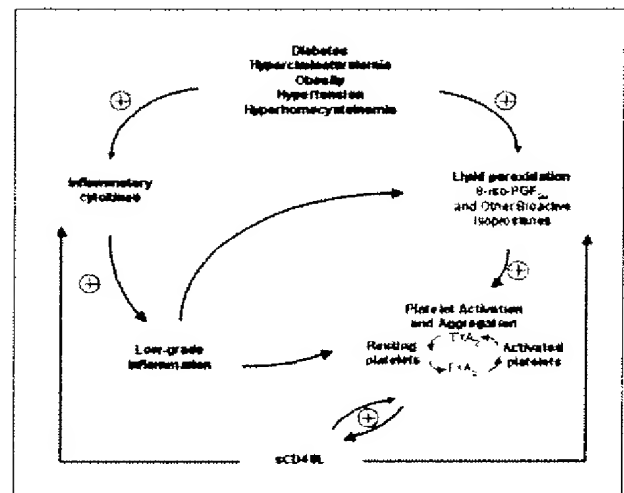


Fig. 2 Role of CD40L in the biochemical mechanisms linking cardiovascular risk factors, inflammation, lipid peroxidation and platelet activation. Potential amplification loops sustaining this chain of events are also shown. PG, prostaglandin; sCD40L, soluble CD40 ligand; TX, thromboxane

state in diabetes [55]. Moreover, elevated plasma sCD40L in patients with diabetes can be reduced by a 1-year multifactorial cardiovascular risk intervention strategy, consisting of ACE inhibitor-based blood pressure control, statin-based lipid lowering, sulphonylurea-based glycaemic control and antiplatelet therapy [55].

The molecular mechanisms linking sCD40L to the accelerated atherosclerosis in diabetes are not yet completely identified. To test the hypothesis that sCD40L is involved in the process of vascular complications in diabetes by triggering a complex group of inflammatory reactions both in vascular endothelial cells and in circulating monocytes/macrophages, we provided evidence in humans that enhanced sCD40L in both T1DM and T2DM is directly responsible for endothelial dysfunction and monocyte activation, demonstrating a positive correlation between sCD40L and ICAM-1, VCAM-1, E-selectin and MCP-1 in diabetic patients [56].

Finally, advanced glycation end-products have been identified as potential triggers of expression and release of sCD40L in diabetic patients [57].

Hypercholesterolaemia

There are several lines of evidence that implicate CD40/CD40L signalling in the vascular pathology associated with hypercholesterolaemia. High cholesterol levels have been associated with enhanced thrombotic risk, possibly related to enhanced persistent platelet activation [58] and thrombin formation [59] observed in this setting. sCD40L is related to cholesterol metabolism as assessed by cholesterol synthesis pathways in patients with moderate hypercholesterolaemia [60]. Patients with moderate hypercholesterolaemia exhibit increased *ex vivo* expression of CD40L and P-selectin on platelets and elevated CD40 expression on monocytes [61]. A larger study [62] showed that in patients with hypercholesterolaemia, increased plasma sCD40L levels have been positively associated with *in vivo* platelet activation, as reflected by plasma P-selectin and urinary 11-dehydro-thromboxane B₂, and with procoagulant state, as reflected by FVIIa and F1+2. Thus, it appears that the CD40/CD40L dyad may provide the mechanistic framework linking inflammation and prothrombotic state in this setting.

A further study was performed to test the hypothesis that CD40L-induced prothrombotic state is mediated by enhancing oxidative stress in the setting of hypercholesterolaemia [63]. Plasma sCD40L, 8-hydroxy-2'-deoxyguanosine, a marker of oxidative stress, and F1+2 levels were significantly higher in patients with hypercholesterolaemia as compared with controls. An additional *in vitro* study showed that CD40L overexpresses TF and increases the thrombin generation rate by an oxidative stress-mediated mechanism that requires activation of NADPH oxidase [64]. This mechanism, in turn, may be responsible for both the accumula-

tion of oxidised LDL within the macrophages, and the prothrombotic state within the plaque. A short-term (3 days) treatment with atorvastatin was able to significantly reduce platelet CD40L and thrombin generation, independently of the lipid-lowering effect of the statin [64].

Cigarette smoking

Cigarette smokers show significantly increased sCD40L levels, surface expression of CD40L on platelets and T cells and of CD40 on monocytes and increased platelet-monocyte aggregates when compared to age- and gender-matched non-smokers [65]. In addition, the surface expression of CD40 on monocytes and of CD40L on platelets correlates with recent intake of nicotine, as assessed by plasma cotinine concentrations and by the number of cigarettes smoked. This finding may represent one of the possible explanations for the increased cardiovascular risk associated with cigarette smoking.

Obesity

We have reported that android obesity is associated with enhanced lipid peroxidation and persistent platelet activation, both improvable by successful weight loss [66]. These abnormalities are driven by inflammatory triggers related to the degree of abdominal adiposity. We found significantly higher plasma CRP levels, in association with enhanced oxidative stress and platelet activation in otherwise healthy women with visceral obesity [66]. Other authors showed increased CD40L levels in patients with severe obesity [67] and that weight loss over a 16-week period of caloric restriction was associated with significant reductions in sCD40L and oxidative stress [68]. In a further study by our group, CD40L plasma levels were elevated in individuals characterised by insulin resistance; however, its relation to increased platelet activation seems to be largely explained by differences in waist-to-hip ratio (WHR) and insulin sensitivity. Successful weight loss over a 12-week period was associated with a statistically significant increase in S₁ and decreases in CD40L, CRP and in urinary 11-dehydro-TXB₂ excretion [69].

Arterial hypertension

Recently, 150 patients with different degree of arterial hypertension were evaluated for the expression of the CD40 system. All patients showed a significant increase of CD40 and CD40L coexpression on platelets as well as sCD40L levels compared with controls, with a slight correlation with blood pressure, suggesting that arterial hypertension is in part an inflammatory disorder [70].

Moreover, nondipper hypertensive patients have enhanced plasma CD40L levels as compared to dippers, with CD40L as the main determinant of IMT [71].

In essential hypertensive patients, microalbuminuria, a recognised marker of preclinical atherosclerosis, is not accompanied by enhanced plasma CD40L concentrations in comparison to hypertensive patients with normoalbuminuria [72].

Metabolic syndrome

Metabolic syndrome is gaining recognition as a multiplex cardiovascular risk factor. The presence of metabolic syndrome is independently associated with elevated sCD40L, CRP and coronary disease severity in CAD patients requiring interventional treatment of stable angina [73]. Patients with metabolic syndrome without cardiovascular disease had significantly higher sCD40L and sP-selectin compared with subjects without metabolic syndrome [74]. Moreover, increased sCD40L levels are related to enhanced thrombotic tendency, as shown by the relationship with prothrombin fragment F 1+2 [75].

Therapeutic implications

Thiazolidinediones

Thiazolidinediones, novel insulin-sensitising antidiabetic agents, act as peroxisome proliferator-activated receptor- γ (PPAR- γ) agonists and have been shown to exhibit anti-inflammatory and antiatherogenic properties [76, 77]. Treatment of diabetic subjects with a thiazolidinedione-type drug results in decreased sCD40L levels [23, 47].

A recent study [78] showed that human bone marrow megakaryocytes and platelets express PPAR- γ , and rosiglitazone is able to prevent thrombin-induced CD40L surface expression and release of CD40L and TXB₂. These results suggest that the reduced plasma levels of CD40L could derive from inhibition of platelet release of CD40L by the dampening effects of the PPAR- γ agonist drug. In this light it is conceivable to think that the antiinflammatory effects of thiazolidinediones may be mediated by modulation of platelet activation, thus identifying in the platelet a new target cell for this class of drugs.

Lipid-lowering drugs

In addition to the well known lipid-lowering effects, statins have been shown to exert anti-inflammatory and antiatherogenic properties [79, 80]. To better address this

issue, several authors have investigated the effects of these drugs on inflammatory markers, including CD40L. Atorvastatin, cerivastatin and simvastatin were able to decrease in a dose-dependent manner the constitutive as well as oxidised LDL- or cytokine-induced expression of the receptor/ligand dyad in human vascular endothelial cells, smooth muscle cells and macrophages [81]. Moreover, statins have been shown to decrease CD40 expression and CD40-related activation of vascular cells, effects partially reversed by the HMG-CoA reductase product mevalonate [82, 83]. Both simvastatin and fenofibrate markedly reduced plasma levels of CD40L, as well as CRP, IL-1 β , and improved endothelium-dependent vascular reactivity in patients with combined hyperlipidaemia [84]. In a substudy in the Atorvastatin versus Simvastatin on Atherosclerosis Progression (ASAP) trial, sCD40L levels were found to be about 27 times higher in 110 asymptomatic patients with familial hypercholesterolaemia as compared to controls [85]; statin therapy over 2 years markedly downregulated sCD40L levels, regardless of the statin used and with no correlation with the degree of cholesterol lowering. Recently, simvastatin, losartan and combined therapy significantly reduced sCD40L in hypercholesterolaemic hypertensive patients, to the greatest extent in subjects with high baseline CD40L levels [86].

In addition to their role in modulating CD40L in the setting of asymptomatic hypercholesterolaemia [81, 87], statins have proven effective in patients with stable CAD [88] and ACS [89, 90]. In patients with ACS enrolled in the Myocardial Ischemia Reduction with Aggressive Cholesterol Lowering (MIRACL) study [91], atorvastatin started within 24–96 h of admission abrogated the risk of recurrent cardiovascular events associated with high sCD40L over 16 weeks of treatment. Interestingly, atorvastatin did not significantly affect sCD40L concentrations over the treatment period. To explain this paradox, the authors speculate that the target of statins also involves downstream events in the CD40L pathway and signalling; in addition, it involves other inflammatory and thrombotic events marked by elevated sCD40L [91]. Given the detrimental effects of CD40L in the pathogenesis and progression of atherothrombosis, the ability of statins to lower the expression of this molecule may have potential therapeutic implications.

Antioxidants

As stated above, several lines of evidence substantiate a role of oxidative stress in enhancing CD40L expression [81, 92]. In particular CD40L downregulation in the setting of a deficit in gp 91 phox, the central core of NADPH oxidase, unravelled a role for O₂ generated by NADPH oxidase as a trigger of CD40L expression [92]. These interest-

ing data paved the way to studies testing the efficacy of antioxidants as modulators of CD40L release by platelets.

Recently, the adjunct of vitamin C *in vitro* to platelets stimulated with collagen, dose dependently inhibited platelet CD40L expression with no effect on platelet aggregation, and vitamin C infusion (1 g per 45 min) to healthy volunteers was associated with a significant and concurrent decrease of platelet O_2^- and CD40L by approximately 70% [93], independently of agonist-induced platelet aggregation.

In addition, CD40L exerts some of its detrimental effects by enhancing release of ROS in platelets [18] and endothelial cells [27], suggesting an additional potential for antioxidants as modulators of CD40L-induced inflammation and platelet activation. In this light, *in vitro* experiments demonstrated inhibition of CD40L-induced clotting system activation by vitamin C [64].

It would be worth testing additional antioxidants, alone or in combination, to further elucidate the efficacy of this novel therapeutic approach in terms of reduction of both CD40L expression and CD40L-induced detrimental cascade.

Antiplatelet agents

As platelets have been shown to be the main contributors to sCD40 shedding, the most obvious approach to down-regulate this mediator seems to be the use of antiplatelet agents such as aspirin. Neither Aukrust et al. [21] nor Cipollone et al. [25] found any significant association between aspirin use and serum levels of sCD40L. These findings are in agreement with the observation that aspirin administration did not attenuate the increased risk of future cardiovascular events associated with high sCD40L levels in healthy women [94]. Aspirin treatment for 7 days in volunteers resulted in a 50% decrease in the release of sCD40L from collagen-induced platelet aggregates *in vitro*, suggesting that TXA_2 is a necessary costimulator of platelets [8]. Recently, to further characterise the platelet origin of plasma CD40L, the dose and time dependence of the effects of aspirin in T2DM was investigated [54]. A randomised, parallel group dose-response study of three different doses (30, 100, 325 mg/day) of aspirin was performed in 18 T2DM patients. Plasma CD40L was significantly reduced by about 40–50% after 7 days of aspirin treatment, with no apparent dose effect, with a slow pattern of recovery over the 10-day wash-out period. The reduction in plasma sCD40L levels as well as the time course of recovery strongly support the hypothesis that in the setting of T2DM both TXA_2 -dependent and -independent mechanisms of platelet activation contribute to enhanced release of sCD40L [54]. Thus, despite aspirin use, more potent platelet inhibitors may be required to inhibit the enhanced release of sCD40L.

Clopidogrel, a potent inhibitor of ADP-induced platelet aggregation, has been reported to block ADP-induced CD40L expression when administered for 7 days to healthy volunteers, leading to the hypothesis that this inhibition may, at least partially, account for the beneficial effects of this drug [95]. Further evidence suggests that clopidogrel lowers levels of CRP and CD40L in patients undergoing PTCA [96, 97]. Patients with stable CAD randomised to 8 weeks of clopidogrel or placebo showed a significant reduction in sCD40L levels in the clopidogrel group [98]. In patients with ACS, a loading dose of clopidogrel (300 mg) attenuated the agonist effects of ADP and thrombin receptor agonist peptide (TRAP) on platelet secretion, aggregation, and formation of platelet-monocyte and platelet-neutrophil conjugates [99]. Plasma levels of soluble CD40L and P-selectin were also significantly reduced and may all contribute to the clinical benefits of the drug in ACS.

A loading dose of clopidogrel followed by 75 mg/day combined with aspirin in patients with UA undergoing PTCA significantly reduced sCD40L levels at 24 h after stenting and during follow-up [100].

GP IIb/IIIa inhibitors

GP IIb/IIIa inhibitors are an important class of drugs used in the management of UA and non-ST elevation MI, as well as in preventing clot formation during PTCA. Because GP IIb/IIIa receptors are a binding site for sCD40L, it has been hypothesised that they might modulate sCD40L release from platelets. Addition of eptifibatide to platelet-rich plasma inhibited sCD40L shedding from activated platelets [8]. Although maximum inhibition of sCD40L release is achieved at clinical dose of these drugs, suboptimal concentration paradoxically potentiated sCD40L release. This finding may help to explain the negative outcome from the orally available GP IIb/IIIa antagonist clinical trials, where suboptimal doses of drugs were typically used.

The aforementioned study by Heeschen and coworkers [22] better defined the clinical relevance of CD40L inhibition by GP IIb/IIIa antagonists, as it was the first to establish a link between sCD40L levels, clinical outcome and the therapeutic tool. A total of 1265 patients with ACS were randomly assigned to receive intravenous abciximab or placebo 18–24 h prior to PTCA. Among patients with the highest sCD40L levels, a significant decrease in the incidence of death or nonfatal MI during the 6-month follow-up was observed in subjects receiving abciximab, as compared to those receiving placebo. In contrast, among patients in the lowest quintiles for sCD40L, no significant benefit was observed with the use of abciximab. Thus, sCD40L levels at baseline may predict both in the short-

and long-term the efficacy of abciximab in improving the rate of cardiovascular events in patients with ACS. The authors did not measure sCD40L levels after abciximab treatment as the trial was not designed to show an effect of the drug on sCD40L plasma levels.

Tirofiban was reported to non-significantly limit the increase in sCD40L after PTCA in patients with stable CAD [101]. Recently, to investigate the effects of abciximab and eptifibatide on sCD40L, 98 ACS patients undergoing PTCA were studied [102]. Eighteen to 24 h after PCI, sCD40L was unchanged in the controls, and reduced 30% in the abciximab-treated group and 9% in the eptifibatide-treated group.

Influence of pre-analytical and analytical factors on sCD40L measurement

Although sCD40L is emerging as a promising marker of thrombotic risk, relatively little attention has been paid to the potential impact of pre-analytical and analytical interferences that may confound interpretation of sCD40L measurements [103–108]. Sampling methods and temperature, in fact, profoundly affect the sCD40L assay [105, 109]. For example, post-harvesting activation of platelets when obtaining serum might account for the higher sCD40L levels found in serum compared to plasma [105]. This finding, together with the close correlation of serum sCD40L with platelet count, supports the possibility that serum sCD40L actually reflects platelet CD40L content [105]. Timing of sample processing in relation to blood collection is another important determinant of sCD40L blood levels [106].

In this light, earlier studies in which serum samples were employed should be interpreted with caution, and the use of serum should be encouraged if the objective is to measure the total pool of sCD40L, including both freely circulating sCD40L and CD40L expressed in platelets, monocytes and T cells [105], but not *in vivo* levels of this cytokine [106]. If this is the case, platelet-depleted citrated plasma samples, processed promptly after collection to minimise *ex vivo* release of sCD40L, should be the preferred sample type [108–110]. Finally, it should be emphasised that sCD40L is actually a pool of free soluble and microparticle-bound (mp-CD40L) forms, the proportion of which is highly variable in each individual [105, 111]. Presently, most protocols used in clinical studies are unable to distinguish either form. Therefore, it is of utmost importance to refine and standardise sCD40L measurement beginning with sample handling techniques such as centrifugation force and time, anticoagulant choice and filtration methods. Once this goal is achieved, we will be able to accurately define the role of sCD40L as a prognostic marker of thrombotic risk.

Concluding remarks

CD40L appears as a multiplayer of several cell types in the inflammatory network.

The peculiarity of CD40L as an inflammatory mediator derived from platelets expands the functional repertoire of platelets from players of haemostasis and thrombosis to powerful amplifiers of inflammation by promoting the release of cytokines and chemokines, cell activation and cell–cell interactions.

Thus, compelling evidence indicating the expression of the CD40/CD40L dyad on many different cell types substantiates the role of this pathway in an extraordinary plethora of biological effects ranging from inflammation, endothelial dysfunction and platelet/coagulative activation. The multifunctional role of CD40L as a simultaneous activator of all these systems further blurs the intricate relationship between such events both in the physiological systems and the pathological derangement occurring in atherothrombosis.

Studies performed so far do not have the power to establish the causal role of CD40L in the pathogenesis of the above-mentioned disease states, but these findings raise the question whether elevated sCD40L levels are a reflection of the pathogenetic role of CD40L in the inflammatory and thrombotic processes or whether they are a mere consequence of the clinical event, namely the result of platelet release after thrombus formation. Even in the latter case, CD40L elevation may constitute a reliable marker of platelet activation and of unstable, prone to rupture plaque, and hopefully, as a predictor of cardiovascular event risk. In this light the availability of an inexpensive, commercially available assay to measure sCD40L levels in several clinical settings may help to identify subjects at major risk of events who may benefit most from more aggressive preventive interventions.

The relationship between sCD40L and global risk assessment remains unclear. In a prospective study increased sCD40L seems to be predictive for future cardiovascular events in healthy women [94]. However, in a large cohort of healthy volunteers, sCD40L levels poorly correlate with both the individual components and the calculated Framingham Coronary Heart Disease Risk Score [112], suggesting the need for long-term follow-up studies to answer the question whether the predictive value of sCD40L is independent of the Framingham based global risk assessment.

Another issue is the association between sCD40L levels, atherosclerotic risk factors and subclinical atherosclerosis. In large and representative multiethnic population studies such as the Dallas Heart studies, sCD40L was not associated with most atherosclerotic risk factors and with subclinical atherosclerosis [113], suggesting that sCD40L is not a reliable tool for screening of early atherosclerosis.

in the general population. It is possible to hypothesise that the validity of sCD40L elevation is proportional to the extent of platelet activation in each setting, but this assumption requires further studies.

The key research priorities for the near future will focus on determining the relevance of sCD40L as a putative therapeutic target. Several studies have already shown the ability to lower CD40L levels by several agents, suggesting its potential role as a reliable tool to monitor the efficacy of antiplatelet, antidiabetic or lipid-lowering drugs. Whether lowering the levels of this molecule translates into an improvement of cardiovascular outcomes remains to be elucidated.

Acknowledgements This research was supported in part by the European Commission FP6 grant (LSHM-CT-2004-005033). This publication reflects only the author's views. The Commission is not liable for any use that may be made of information herein. We are indebted to Dr Laura Di Benedetto for expert editorial assistance.

References

1. Armitage RJ, Fanslow WC, Strockbine L et al (1992) Molecular and biological characterization of a murine ligand for CD40. *Nature* 357:80–82
2. Lederman S, Yellin MJ, Krichevsky A et al (1992) Identification of a novel surface protein on activated CD4+ T cells that induces contact-dependent B cell differentiation (help). *J Exp Med* 175:1091–1101
3. Gauchat JF, Henchoz S, Mazzei G et al (1993) Induction of human IgE synthesis in B cells by mast cells and basophils. *Nature* 365:340–343
4. Henn V, Slupsky JR, Gräfe M et al (1998) CD40 ligand on activated platelets triggers an inflammatory reaction of endothelial cells. *Nature* 391:591–594
5. Schönbeck U, Libby P (2001) The CD40/CD154 receptor/ligand dyad. *Cell Mol Life Sci* 58:4–43
6. Henn V, Steinbach S, Büchner K et al (2001) The inflammatory action of CD40 ligand (CD154) expressed on activated human platelets is temporally limited by coexpressed CD40. *Blood* 98:1047–1054
7. Andre P, Prasad KS, Denis CV et al (2002) CD40L stabilizes arterial thrombi by a $\beta 3$ integrin-dependent mechanism. *Nat Med* 8:247–252
8. Nannizzi-Alaimo L, Alves VL, Phillips DR (2003) Inhibitory effects of glycoprotein IIb/IIIa antagonists and aspirin on the release of soluble CD40 ligand during platelet stimulation. *Circulation* 107:1123–1128
9. Otterdal K, Pedersen TM, Solum NO (2004) Release of soluble CD40 ligand after platelet activation: studies on the solubilization phase. *Thromb Res* 114:167–177
10. Furman MI, Krueger LA, Linden MD et al (2004) Release of soluble CD40L from platelets is regulated by glycoprotein IIb/IIIa and actin polymerization. *J Am Coll Cardiol* 43:2326–2328
11. Inwald DP, McDowall A, Peters MJ et al (2003) CD40 is constitutively expressed on platelets and provides a novel mechanism for platelet activation. *Circ Res* 92:1041–1048
12. Prasad KS, Andre P, He M et al (2003) Soluble CD40 ligand induces $\beta 3$ integrin tyrosine phosphorylation and triggers platelet activation by outside-in signaling. *Proc Natl Acad Sci U S A* 100:12367–12371
13. Furman MI, Krueger LA, Linden MD et al (2005) GPIIb-IIIa antagonists reduce thromboinflammatory processes in patients with acute coronary syndromes undergoing percutaneous coronary intervention. *J Thromb Haemost* 3:312–320
14. Lutgens E, Daemen MJ (2002) CD40–CD40L interactions in atherosclerosis. *Trends Cardiovasc Med* 12:27–32
15. Zhou L, Stordeur P, de Lavareille A et al (1998) CD40 engagement on endothelial cells promotes tissue factor-dependent procoagulant activity. *Thromb Haemost* 79:1025–1028
16. Schönbeck U, Mach F, Sukhova GK et al (2000) CD40 ligation induces tissue factor expression in human vascular smooth muscle cells. *Am J Pathol* 156:7–14
17. Lindmark E, Tenno T, Siegbahn A (2000) Role of platelet P-selectin and CD40 ligand in the induction of monocytic tissue factor expression. *Arterioscler Thromb Vasc Biol* 20:2322–2328
18. Chakrabarti S, Varghese S, Vitseva O et al (2005) CD40 ligand influences platelet release of reactive oxygen intermediates. *Arterioscler Thromb Vasc Biol* 25:2428–2434
19. Zirlik A, Maier C, Gerdes N et al (2007) CD40 ligand mediates inflammation independently of CD40 by interaction with Mac-1. *Circulation* 115:1571–1580
20. Chai H, Yan S, Wang H et al (2006) CD40 ligand increases expression of its receptor CD40 in human coronary artery endothelial cells. *Surgery* 140:236–242
21. Aukrust P, Muller F, Ueland T et al (1999) Enhanced levels of soluble and membrane-bound CD40 ligand in patients with unstable angina. Possible reflection of T lymphocyte and platelet involvement in the pathogenesis of acute coronary syndromes. *Circulation* 100:614–620
22. Heeschen C, Dimmeler S, Hamm CW et al; CAPTURE Study Investigators (2003) Soluble CD40 ligand in acute coronary syndromes. *N Engl J Med* 348:1104–1111
23. Marx N, Imhof A, Froelich J et al (2003) Effect of rosiglitazone treatment on soluble CD40L in patients with type 2 diabetes and coronary artery disease. *Circulation* 107:1954–1957
24. Nannizzi-Alaimo L, Rubenstein MH, Alves VL et al (2002) Cardiopulmonary bypass induces release of soluble CD40 ligand. *Circulation* 105:2849–2854
25. Cipollone F, Ferri C, Desideri G et al (2003) Preprocedural level of soluble CD40L is predictive of enhanced inflammatory response and restenosis after coronary angioplasty. *Circulation* 108:2776–2782
26. Turker S, Gencer S, Akdeniz B et al (2006) Usefulness of preprocedural soluble CD40 ligand for predicting restenosis after percutaneous coronary intervention in patients with stable coronary artery disease. *Am J Cardiol* 97:198–202
27. Urbich C, Dermach E, Aicher A et al (2002) CD40 ligand inhibits endothelial cell migration by increasing production of endothelial reactive oxygen species. *Circulation* 106:981–986
28. Varo N, de Lemos JA, Libby P et al (2003) Soluble CD40L: risk prediction after acute coronary syndromes. *Circulation* 108:1149–1152

29. Antoniades C, Tousoulis D, Vasiliadou C et al (2006) Genetic polymorphisms of platelet glycoprotein Ia and the risk for premature myocardial infarction: effects on the release of sCD40L during the acute phase of premature myocardial infarction. *J Am Coll Cardiol* 47:1959–1966
30. Malarstig A, Lindahl B, Wallentin L, Siegbahn A (2006) Soluble CD40L levels are regulated by the -3459 A>G polymorphism and predict myocardial infarction and the efficacy of antithrombotic treatment in non-ST elevation acute coronary syndrome. *Arterioscler Thromb Vasc Biol* 26:1667–1673
31. Blake GJ, Ostfeld RJ, Yucel EK et al (2003) Soluble CD40 ligand levels indicate lipid accumulation in carotid atheroma: an in vivo study with high resolution MRI. *Arterioscler Thromb Vasc Biol* 23:e11–e14
32. Novo S, Basili S, Tantillo R et al (2005) Soluble CD40L and cardiovascular risk in asymptomatic low-grade carotid stenosis. *Stroke* 36:673–675
33. Cha JK, Jeong MH, Jang JY et al (2003) Serial measurement of surface expressions of CD63, P-selectin and CD40 ligand on platelets in atherosclerotic ischemic stroke. A possible role of CD40 ligand on platelets in atherosclerotic ischemic stroke. *Cerebrovasc Dis* 16:376–382
34. Garlichs CD, Kozina S, Fateh-Moghadam S et al (2003) Upregulation of CD40-CD40 ligand (CD154) in patients with acute cerebral ischemia. *Stroke* 34:1412–1418
35. Grau AJ, Lichy C (2003) Editorial comment: stroke and the CD40-CD40 ligand system: at the hinge between inflammation and thrombosis. *Stroke* 34:1417–1418
36. Desideri G, Cipollone F, Necozone S et al (2006) Enhanced soluble CD40 ligand and Alzheimer's disease: evidence of a possible pathogenetic role. *Neurobiol Aging* Nov 22 [Epub ahead of print]
37. Lutgens E, Cleutjens KB, Heeneman S et al (2000) Both early and delayed anti-CD40L antibody treatment induces a stable plaque phenotype. *Proc Natl Acad Sci U S A* 97:7464–7469
38. Blann AD, Tan KT, Tayebjee MH et al (2005) Soluble CD40L in peripheral artery disease. Relationship with disease severity, platelet markers and the effects of angioplasty. *Thromb Haemost* 93:578–583
39. Herve P, Humbert M, Sitbon O et al (2001) Pathobiology of pulmonary hypertension. The role of platelets and thrombosis. *Clin Chest Med* 22:451–458
40. Damas JK, Otterdal K, Yndestad A et al (2004) Soluble CD40 ligand in pulmonary arterial hypertension: possible pathogenic role of the interaction between platelets and endothelial cells. *Circulation* 110:999–1005
41. Stumpf C, Lehner C, Eskafi S et al (2003) Enhanced levels of CD154 (CD40 ligand) on platelets in patients with chronic heart failure. *Eur J Heart Fail* 5:629–637
42. Ueland T, Aukrust P, Yndestad A et al (2005) Soluble CD40 ligand in acute and chronic heart failure. *Eur Heart J* 26:1101–1107
43. Gaede P, Vedel P, Larsen N et al (2003) Multifactorial intervention and cardiovascular disease in patients with type 2 diabetes. *N Engl J Med* 348:383–393
44. Sjöholm A, Nystrom T (2005) Endothelial inflammation in insulin resistance. *Lancet* 365:610–612
45. Davi G, Ciabattini G, Consoli A et al (1999) In vivo formation of 8-iso-PGF_{2a} and platelet activation in diabetes mellitus: effects of improved metabolic control and vitamin E supplementation. *Circulation* 99:224–229
46. Davi G, Catalano I, Averna M et al (1990) Thromboxane biosynthesis and platelet function in type II diabetes mellitus. *N Engl J Med* 322:1769–1774
47. Varo N, Vicent D, Libby P et al (2003) Elevated plasma levels of the atherogenic mediator soluble CD40 ligand in diabetic patients. A novel target of thiazolidinediones. *Circulation* 107:2664–2669
48. Jinchuan Y, Zonggui W, Jinming C et al (2004) Upregulation of CD40-CD40 ligand system in patients with diabetes mellitus. *Clin Chim Acta* 339:85–90
49. Cabeza N, Li Z, Schulz C et al (2004) Surface expression of collagen receptor Fc receptor-gamma/glycoprotein VI is enhanced on platelets in type 2 diabetes and mediates release of CD40 ligand and activation of endothelial cells. *Diabetes* 53:2117–2121
50. Yngen M, Ostenson CG, Hu H et al (2004) Enhanced P-selectin expression and increased soluble CD40 ligand in patients with type 1 diabetes mellitus and microangiopathy: evidence for platelet hyperactivity and chronic inflammation. *Diabetologia* 47:537–540
51. Targher G, Zoppini G (2004) Soluble CD40L in young type 1 diabetic individuals without clinical microvascular and macrovascular complications. *Diabetes Care* 27:1236–1237
52. Katakami N, Kaneto H, Matsuhisa M et al (2006) Association of soluble CD40 ligand with carotid atherosclerosis in Japanese type 1 diabetic patients. *Diabetologia* 49:1670–1676
53. Harding SA, Sommerfield AJ, Sarma J et al (2004) Increased CD40 ligand and platelet-monocyte aggregates in patients with type 1 diabetes mellitus. *Atherosclerosis* 176:321–325
54. Santilli F, Davi G, Consoli A et al (2006) Thromboxane-dependent CD40 ligand release in type 2 diabetes mellitus. *J Am Coll Cardiol* 47:391–397
55. Lim HS, Blann AD, Lip GY (2004) Soluble CD40 ligand, soluble P-selectin, interleukin-6 and tissue factor in diabetes mellitus. Relationship to cardiovascular disease and risk factor intervention. *Circulation* 109:2524–2528
56. Cipollone F, Chiarelli F, Davi G et al (2005) Enhanced soluble CD40 ligand contributes to endothelial cells dysfunction in vitro and monocyte activation in patients with diabetes mellitus: effect of improved metabolic control. *Diabetologia* 48:1216–1224
57. Varo N, Libby P, Nuzzo R et al (2005) Elevated release of sCD40L from platelets of diabetic patients by thrombin, glucose and advanced glycation end products. *Diab Vasc Dis Res* 2:81–87
58. Davi G, Averna M, Catalano I et al (1992) Increased thromboxane biosynthesis in type IIa hypercholesterolemia. *Circulation* 85:1792–1798
59. Davi G, Ganci A, Averna M et al (1995) Thromboxane biosynthesis, neutrophil and coagulative activation in type IIa hypercholesterolemia. *Thromb Hemost* 74:247–251
60. Luomala M, Paiva H, Laaksonen R et al (2006) Plasma-soluble CD40 is related to cholesterol metabolism in patients with moderate hypercholesterolemia. *Scand Cardiovasc J* 40:280–284
61. Garlichs CD, John S, Schmeisser A et al (2001) Upregulation of CD40 and CD40 ligand (CD154) in patients with moderate hypercholesterolemia. *Circulation* 104:2395–2400
62. Cipollone F, Mezzetti A, Porreca E et al (2002) Association between enhanced soluble CD40L and prothrombotic state in hypercholesterolemia: effects of statin therapy. *Circulation* 106:399–402

63. Sanguigni V, Ferro D, Pignatelli P et al (2005) CD40 ligand enhances monocyte tissue factor expression and thrombin generation via oxidative stress in patients with hypercholesterolemia. *J Am Coll Cardiol* 45:35–42
64. Sanguigni V, Pignatelli P, Lenti L et al (2005) Short-term treatment with atorvastatin reduces platelet CD40 ligand and thrombin generation in hypercholesterolemic patients. *Circulation* 111:412–419
65. Harding SA, Sarma J, Josephs DH et al (2004) Upregulation of the CD40/CD40 ligand dyad and platelet-monocyte aggregation in cigarette smokers. *Circulation* 109:1926–1929
66. Davi G, Guagnano MT, Ciabattini G et al (2002) Platelet activation in obese women: role of inflammation and oxidant stress. *JAMA* 288:2008–2014
67. Guldiken B, Guldiken S, Turgut B et al (2007) Serum osteoprotegerin levels in patients with acute atherothrombotic stroke and lacunar infarct. *Thromb Res* 120:511–516
68. Desideri G, Ferri C (2003) Effects of obesity and weight loss on soluble CD40L levels. *JAMA* 289:1781–1782
69. Basili S, Pacini G, Guagnano MT et al (2006) Insulin resistance as a determinant of platelet activation in obese women. *J Am Coll Cardiol* 48:2531–2538
70. Yan JC, Ma GS, Wu ZG et al (2005) Increased levels of CD40-CD40 ligand system in patients with essential hypertension. *Clin Chim Acta* 355:191–196
71. Desideri G, Cipollone F, Valeri L et al (2007) Enhanced plasma soluble CD40 ligand levels in essential hypertensive patients with blunted nocturnal blood pressure decrease. *Am J Hypertens* 20:70–76
72. Tsioufis C, Dimitriadis K, Taxiarchou E et al (2006) Diverse associations of microalbuminuria with C-reactive protein, interleukin-18 and soluble CD 40 ligand in male essential hypertensive subjects. *Am J Hypertens* 19:462–466
73. Lee WL, Lee WJ, Chen YT et al (2006) The presence of metabolic syndrome is independently associated with elevated serum CD40 ligand and disease severity in patients with symptomatic coronary artery disease. *Metabolism* 55:1029–1034
74. Gokulakrishnan K, Deepa R, Mohan V, Gross MD (2006) Soluble P-selectin and CD40L levels in subjects with prediabetes, diabetes mellitus, and metabolic syndrome-the Chennai Urban Rural Epidemiology Study. *Metabolism* 55:237–242
75. Angelico F, Alessandri C, Ferro D et al (2006) Enhanced soluble CD40L in patients with the metabolic syndrome: Relationship with in vivo thrombin generation. *Diabetologia* 49:1169–1174
76. Marx N (2002) Peroxisome proliferator-activated receptor gamma and atherosclerosis. *Curr Hypertens Rep* 4:71–77
77. Jiang C, Ting AT, Seed B (1998) PPAR- γ agonists inhibit production of monocyte inflammatory cytokines. *Nature* 391:82–86
78. Akbiyik F, Ray DM, Gettings KF et al (2004) Human bone marrow megakaryocytes and platelets express PPAR- γ , and PPAR- γ agonists blunt platelet release of CD40 ligand and thromboxanes. *Blood* 104:1361–1368
79. Blake GJ, Ridker PM (2000) Are statins anti-inflammatory? *Curr Control Trial Cardiovasc Med* 1:161–165
80. Shishehbor MH, Brennan ML, Aviles RJ et al (2003) Statins promote potent systemic antioxidant effects through specific inflammatory pathways. *Circulation* 108:426–431
81. Schönbeck U, Gerdes N, Varo N et al (2002) Oxidized low-density lipoprotein augments and 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors limit CD40 and CD40L expression in human vascular cells. *Circulation* 106:2888–2893
82. Wagner AH, Gebauer M, Guldenzoph B, Hecker M (2002) 3-Hydroxy-3-methylglutaryl coenzyme A reductase-independent inhibition of CD40L expression by atorvastatin in human endothelial cells. *Arterioscler Thromb Vasc Biol* 22:1784–1789
83. Mulhaupt F, Matter CM, Kwak BR et al (2003) Statins (HMG-CoA reductase inhibitors) reduce CD40 expression in human vascular cells. *Cardiovasc Res* 59:755–766
84. Wang TD, Chen WJ, Lin JW et al (2003) Efficacy of fenofibrate and simvastatin on endothelial function and inflammatory markers in patients with combined hyperlipidemia: relations with baseline lipid profiles. *Atherosclerosis* 170:315–323
85. Semb AG, van Wissen S, Ueland T et al (2003) Raised serum levels of soluble CD40 ligand in patients with familial hypercholesterolemia: downregulatory effect of statin therapy. *J Am Coll Cardiol* 41:275–279
86. Han SH, Koh KK, Quon MJ et al (2007) The effects of simvastatin, losartan, and combined therapy on soluble CD40 ligand in hypercholesterolemic, hypertensive patients. *Atherosclerosis* 190:205–211
87. Hwang YS, Tsai WC, Lu YH et al (2004) Effect of atorvastatin on the expression of CD40 ligand and P-selectin on platelets in patients with hypercholesterolemia. *Am J Cardiol* 94:364–366
88. Alber HF, Frick M, Suessenbacher A et al (2006) Effect of atorvastatin on circulating proinflammatory T-lymphocyte subsets and soluble CD40 ligand in patients with stable coronary artery disease – a randomized, placebo-controlled study. *Am Heart J* 151:139
89. Li J, Zhao SP, Peng DQ et al (2004) Early effect of pravastatin on serum soluble CD40L, matrix metalloproteinase-9, and C-reactive protein in patients with acute myocardial infarction. *Clin Chem* 50:1696–1699
90. Mitsios JV, Papathanasiou AI, Rodis FI et al (2004) Atorvastatin does not affect the antiplatelet potency of clopidogrel when it is administered concomitantly for 5 weeks in patients with acute coronary syndromes. *Circulation* 109:1335–1338
91. Kinlay S, Schwartz GG, Olsson AG et al (2004) Myocardial Ischemia Reduction with Aggressive Cholesterol Lowering (MIRACL) Study Investigators. Effect of atorvastatin on risk of recurrent cardiovascular events after an acute coronary syndrome associated with high soluble CD40 ligand in the Myocardial Ischemia Reduction with Aggressive Cholesterol Lowering (MIRACL) Study. *Circulation* 110:386–391
92. Pignatelli P, Sanguigni V, Lenti L et al (2004) gp91phox-dependent expression of platelet CD40 ligand. *Circulation* 110:1326–1329
93. Pignatelli P, Sanguigni V, Paola SG et al (2005) Vitamin C inhibits platelet expression of CD40 ligand. *Free Radic Biol Med* 38:1662–1666
94. Schönbeck U, Varo N, Libby P et al (2001) Soluble CD40L and cardiovascular risk in women. *Circulation* 104:2266–2268

95. Hermann A, Rauch BH, Braun M et al (2001) Platelet CD40 ligand (CD40L)-subcellular localization, regulation of expression, and inhibition by clopidogrel. *Platelets* 12:74–82
96. Bhatt DL, Topol EJ (2003) Scientific and therapeutic advances in antiplatelet therapy. *Nat Rev Drug Discov* 2:15–28
97. Quinn MJ, Bhatt DL, Zidar F et al (2004) Effect of clopidogrel pretreatment on inflammatory marker expression in patients undergoing percutaneous coronary intervention. *Am J Cardiol* 93:679–684
98. Azar RR, Kassab R, Zoghbi A et al (2006) Effects of clopidogrel on soluble CD40 ligand and on high-sensitivity C-reactive protein in patients with stable coronary artery disease. *Am Heart J* 151:521.e1–521.e4
99. Xiao Z, Theroux P (2004) Clopidogrel inhibits platelet-leukocyte interactions and thrombin receptor agonist peptide-induced platelet activation in patients with an acute coronary syndrome. *J Am Coll Cardiol* 43:1982–1988
100. Yip HK, Chang LT, Sun CK et al (2006) Impact of clopidogrel on suppression of circulating levels of soluble CD40 ligand in patients with unstable angina undergoing coronary stenting. *Am J Cardiol* 97:192–194
101. Azar RR, Badaoui G, Sarkis A et al (2005) Effects of tirofiban and statins on high-sensitivity C-reactive protein, interleukin-6, and soluble CD40 ligand following percutaneous coronary interventions in patients with stable coronary artery disease. *Am J Cardiol* 95:236–240
102. Mo VY, De Lemos JA (2004) Individualizing therapy in acute coronary syndromes: using a multiple biomarker approach for diagnosis, risk stratification, and guidance of therapy. *Curr Cardiol Rep* 6:273–278
103. Conde JD, Kleiman NS (2003) Soluble CD40 ligand in acute coronary syndromes. *N Engl J Med* 384:2575–2576
104. Berczki D, Nagy E, Pal A et al (2003) Should soluble CD40 ligand be measured from serum or plasma samples? *Arterioscler Thromb Vasc Biol* 23:1129–1130
105. Ahn ER, Lander G, Jy W et al (2004) Differences of soluble CD40L in sera and plasma: implications on CD40L assay as a marker of thrombotic risk. *Thromb Res* 114:143–148
106. Thom J, Gilmore G, Yi Q et al (2004) Measurement of soluble P-selectin and soluble CD40 ligand in serum and plasma. *J Thromb Haemost* 2:2067–2069
107. Halldorsdottir AM, Stoker J, Porche-Sorbet R, Eby CS (2005) Soluble CD40 ligand measurement inaccuracies attributable to specimen type, processing time, and ELISA method. *Clin Chem* 51:1054–1057
108. Varo N, Nuzzo R, Natal C et al (2006) Influence of pre-analytical and analytical factors on soluble CD40L measurements. *Clin Sci (Lond)* 111:341–347
109. Weber M, Rabenau B, Stanisch M et al (2006) Influence of sample type and storage conditions on soluble CD40 ligand assessment. *Clin Chem* 52:888–891
110. Lessiani G, Falco A, Dragani A et al (2006) Polycythemia vera and endothelial dysfunction: role of CD40 ligand. *Atherosclerosis Suppl* 7:88
111. Anand SX, Viles-Gonzalez JF, Badimon JJ et al (2003) Membrane-associated CD40L and sCD40L in atherothrombotic disease. *Thromb Haemost* 90:377–384
112. Verma S, Wang CH, Li SH et al; FATE Investigators (2005) The relationship between soluble CD40 ligand levels and Framingham coronary heart disease risk score in healthy volunteers. *Atherosclerosis* 182:361–365
113. de Lemos JA, Zirlik A, Schonbeck U et al (2005) Associations between soluble CD40 ligand, atherosclerosis risk factors, and subclinical atherosclerosis: results from the Dallas Heart Study. *Arterioscler Thromb Vasc Biol* 25:2192–2196

Exhibit 2: Borchert et al., “Review of Pleiotropic Effects of Peroxisome Proliferator-Activated Receptor γ Agonists on Platelet Function,” *Diabetes Technology & Therapeutics* 9(5):410-420 (2007)

Review

Review of the Pleiotropic Effects of Peroxisome Proliferator-Activated Receptor γ Agonists on Platelet Function

M. BORCHERT, B.Sc.,¹ T. SCHÖNDORF, Ph.D.,^{1,2} G. LÜBBEN, M.D.,³ T. FORST, M.D.,¹
and A. PFÜTZNER, M.D., Ph.D.^{1,4}

ABSTRACT

The primary target receptor for thiazolidinediones (TZDs) or peroxisome proliferator-activated receptor γ (PPAR γ) agonists is a transcription factor in the nucleus of adipocytes and other metabolically active cells, where they improve insulin sensitivity and glucose utilization. TZDs are also able to modify gene expression in macrophages, smooth muscle cells, and endothelial cells. Although PPAR γ is considered to be a nuclear receptor, enucleate platelets also highly express this receptor. The aim of this review is to present the current understanding of a direct or indirect effect of TZDs on platelet function. By means of a comprehensive literature search (January 1990–June 2006), publications were obtained that contained specific information about in vitro and in vivo effects of TZDs on platelet function. The effects were studied for different risk biochemical markers, i.e., proteins found to be elevated in the state of procoagulant inflammation and endothelial dysfunction. Improvement of platelet function was reported for all TZDs—troglitazone, pioglitazone, and rosiglitazone. The described effects included reduction of platelet aggregation, suppression of thrombin-induced protein kinase C- α and - β activation, decrease in plasma P-selectin and platelet P-selectin expression, increase in nitric oxide production, inhibition of the Rho/Rho kinase pathway, and inhibition of tissue factor- and platelet-activating factor-induced morphological changes in macrophages. These findings appeared in parallel with reduction of the plasma concentrations of pro-inflammatory risk markers. TZDs seem to have a direct pleiotropic positive influence on platelet function and coagulation and may be helpful in treating the prothrombotic state observed in patients with type 2 diabetes and metabolic syndrome.

¹Institute for Clinical Research and Development, Mainz, Germany.

²University of Cologne, Medical Center, Cologne, Germany.

³Takeda Pharma, Aachen, Germany.

⁴Department of Applied Natural Sciences, University of Applied Science, Rheinbach, Germany.
The first two authors contributed equally to this work.

INTRODUCTION

TYPE 2 DIABETES MELLITUS is considered to be one of the major risk factors involved in the development of atherosclerosis. Atherosclerosis is characterized by the infiltration of mononuclear leukocytes into the intima, local expansion of vascular smooth muscle cells (VSMCs), and accumulation of extracellular matrix.^{1,2} Its sequelae—ischemic macrovascular events such as coronary artery disease, stroke, transient ischemic attack, and peripheral artery disease³—ultimately lead to the high prevalence of macrovascular morbidity and mortality among patients suffering from diabetes (75%).^{4,5} The clustering of stand-alone risk factors in diabetic disorders for cardiovascular complications can also be regarded the basis for the high prevalence of microvascular comorbidities such as retinopathy, nephropathy, and neuropathy.⁶

Patients comprising the different metabolic phenotypes observed as the metabolic syndrome must be considered at high risk for atherosclerosis.⁷ Recent reports clearly show a prevalent pro-inflammatory state by creating a proliferative and prothrombotic milieu^{3,8} in patients with the metabolic syndrome or diabetes.⁹ This condition must be considered as the dominating link to atherogenesis as well as the increased rate of cardiovascular events in this population. The metabolic abnormalities encountered in diabetes such as obesity, insulin resistance, hypertension, impaired glucose tolerance (or diabetes), hyperinsulinemia, and dyslipidemia [characterized by elevated concentrations of triglyceride and low-density lipoprotein (LDL), and by low concentrations of high-density lipoprotein], render arteries susceptible to atherosclerosis.¹⁰

In this context, various reports draw a picture of the vascular state in diabetes with characteristic detrimental alterations such as a dysfunctional endothelium, an impaired coagulation cascade,^{11,12} activated and hyperreactive platelets,^{11,13} and aberrant fibrinolysis.^{14,15} The accumulation of abnormalities in carbohydrate and lipid metabolism is capable of altering the functional properties of multiple cell types, including adipocytes, leukocytes, endothelial cells, and platelets,² as well as VSMCs.

Micro- and macrovascular complications are encountered by physicians as the principal cause of death and disability in people with diabetes.¹⁶ Such complications indicate the need for pathophysiologically oriented therapeutic strategies that should not just target treatment of hyperglycemia but also reduce insulin resistance and β -cell dysfunction. The high incidence of diabetes and the devastating reports of the expected future increase into epidemic dimensions drive investigations to explore the relationship of the observed metabolic and cardiovascular disorders as well as to facilitate the selection of an appropriate therapy, as can be observed by numerous scientific publications appearing every month. The thiazolidinediones (TZDs) are potential candidates for this approach because of their multiple beneficial effects on different cell types, their positive influence on the disturbed metabolism of patients with diabetes, and their potential antithrombotic effects. This review aims at providing an overview of the latest consolidated findings about the pleiotropic effects of TZD on platelets and their activation from recent clinical trials.

PLATELETS AND THEIR ACTIVATION

Platelets are essential to hemostasis, and their dysfunction may cause acute vascular events as a consequence of increased thrombus formation. They are anuclear cells derived from megakaryocytes in the bone marrow, but contain small amounts of mRNA and retain the necessary enzymes for mRNA processing.¹⁷ When activated, platelets release a variety of pro-inflammatory hormones and cytokines, such as prostaglandin E₂, transforming growth factor β , thromboxane A₂ (TXA₂), CD40 ligand (CD40L), interleukin (IL)-1 β , and plasminogen activator inhibitor-1 (PAI-1).¹⁸ Hemostasis and the pro-inflammatory state are both characterized by a two-phase platelet aggregation, mediated via the membrane-bound glycoprotein complex (glycoprotein IIb/IIIa receptor). Reversible blood clots agglomerate because of augmented platelet adhesion after contact with free collagen fibrils (e.g., following cell wall rupture) or with immune complexes. Platelet-derived prostaglandins induce irreversible

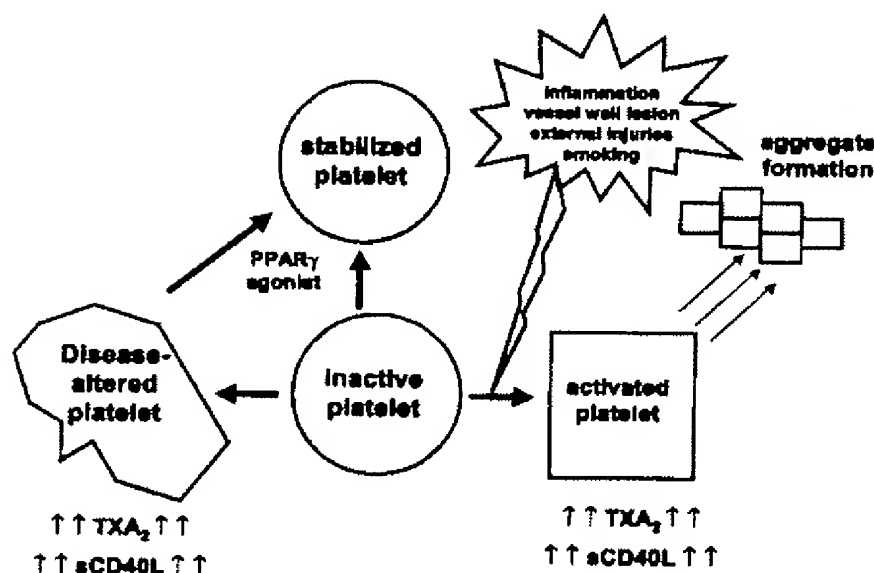


FIG. 1. Cellular effects of PPAR γ activation on platelets.

blood coagulation. The final product of the coagulation cascade, fibrin, is generated via thrombin activation in concert with activated platelet factors such as fibrinogen, von Willebrand factor, and several other plasma factors.¹⁹ A graphic representation of the physiologic features of platelets including the cellular effects of peroxisome proliferator-activated receptor γ (PPAR γ) activation is provided in Figure 1.

Erosions of the arterial intima may expose subendothelial collagen to blood, which will lead to platelet adhesion and activation. P-selectin expression on platelet surface also facilitates adherence to leukocytes and endothelial cells and enhances the expression of tissue factor on monocytes. Enzymatically shed from platelet surface, soluble P-selectin is considered to be a marker of platelet activation and was shown to be associated with carotid atherosclerosis.²⁰ Platelets may also be activated directly by platelet-activating factor (PAF).²¹ The occurrence of *in vivo* platelet activation has been reported in type IIa hypercholesterolemic patients, suggesting that high LDL levels, through changes in the composition of platelet membrane phospholipids and cholesterol, may increase platelet reactivity with enhanced TXA₂

biosynthesis.² Mildly oxidized LDL has also been reported to induce platelet aggregation through activation of phospholipase A₂ and enhanced TXA₂ secretion from platelets, resulting in release of several potent growth factors.² The release of these growth factors, such as platelet-derived growth factor, have mitogenic effects on the fibroblasts in close vicinity to the formed thrombus, and also on the proliferation of other cells in the process of vessel wall remodeling. Mildly oxidized LDL in combination with TXA₂ may also directly stimulate vessel cell proliferation.

The insulin-induced activities include inhibition of platelet aggregation by prompting the synthesis of nitric oxide (NO) in platelets and prostacyclin in endothelial cells. Furthermore, insulin up-regulates prostacyclin receptors and down-regulates α_2 -adrenergic receptors in platelets, thereby amplifying the inhibition of platelet aggregation. Insulin also releases tissue plasminogen activator, a potent thrombolytic enzyme, from the platelet membrane, which dissolves the formed thrombus leading to the resumption of normal blood circulation.²² Platelets from obese subjects and patients with type 2 diabetes are resistant to the ability of insulin or acetylsalicylic acid to counteract

platelet aggregation.^{23,24} Under hyperglycemic conditions in insulin resistance or diabetes mellitus, platelets are considered to become hyperreactive.⁶ They contribute, in concert with increased cytokine secretion (CD40), to cardiovascular risk as a pro-inflammatory and pro-coagulant factor. CD40L is normally not expressed on platelet surfaces. In an activated state, however, it is expressed very rapidly. Vascular cell CD40 surface protein interacts with CD40L of platelets and induces the expression of adhesion molecules and the release of inflammatory cytokines (such as IL-6 by monocytes), as well as the procoagulant tissue factor. CD40L is eventually cleaved from the platelet surface and released into circulation as soluble CD40L (sCD40L), which retains its biological activity. Therefore, like platelet-derived soluble P-selectin, sCD40L may reflect platelet activation, and it is considered a potential target biomarker for successful treatment of cardiovascular disease.^{25,26}

PLATELET RESPONSE TO PPAR γ ACTIVATION: IN VITRO RESULTS

Platelets do not have a nucleus, but it was recently discovered that they contain large amounts of PPAR γ protein, while no mRNA for this protein became apparent.²⁷ The platelet PPAR γ protein, which is most likely derived from the parent megakaryocyte, has DNA binding ability that can be enhanced by PPAR γ agonists. In vitro, physiological levels of PPAR γ agonists can reduce TXA $_2$ release and expression and release of CD40 ligands from thrombin-activated platelets.^{27,28} Pretreatment of human umbilical vein endothelial cells with pioglitazone for 60 min significantly decreased CD40 and CD40L expression induced by oxidized LDL in a concentration-dependent manner.²⁹

When thromboxane production was assessed in human erythroleukemia cells and human platelets by means of gas chromatography/selected ion monitoring, the PPAR γ agonist troglitazone reduced the TXA $_2$ production and also reduced thrombin-induced TXA $_2$ release from platelets in vitro.³⁰ Activation of the PAF, a key molecule in inflammation, is responsible for cytoskeletal reorganiza-

tion in macrophages to increase cell motility. Platelets from patients with type 2 diabetes have lost their responsiveness to insulin.²⁴ As a result, platelets exert increased adhesion, aggregation, and procoagulatory activity after contact with collagen. Pioglitazone inhibits actions of PAF in macrophages by inducing PAF-acetylhydrolase, the enzyme that inactivates the PAF molecule by degradation.³¹

The proliferation of VSMCs is a known response to arterial injury that is an important part of the process of restenosis and atherosclerosis. Troglitazone reduces VSMC migration.³² The antiproliferative effects of three PPAR γ agonists—troglitazone, rosiglitazone, and pioglitazone—on VSMCs derived from the three vascular beds used for coronary artery bypass grafting (the internal mammary and radial artery and saphenous veins) were investigated in vitro by cell counting and cell cycle studies by western blotting for phosphorylated retinoblastoma protein. All three TZDs showed inhibitory potency toward cell proliferation.³³ Table 1 summarizes the results from in vitro studies.

PLATELET RESPONSE TO PPAR γ ACTIVATION—RESULTS FROM ANIMAL STUDIES

Next to cell culture experiments, animal studies provided further evidence for a potential antithrombotic effect of PPAR γ agonists (Table 2). In a murine model, troglitazone and pioglitazone treatment increased platelet count, decreased platelet-associated antibodies, and increased the survival period of antibody-sensitized erythrocytes.⁴³ When Sprague-Dawley rats were fed chow mixed with pioglitazone in comparison to placebo, active drug feeding delayed the time to occlusive thrombus formation by 40% without affecting the weight of the thrombus. ADP- as well as arachidonic acid-induced platelet aggregation was inhibited by pioglitazone feeding, which also up-regulated the aortic expression of constitutive NO synthase (cNOS) and thrombomodulin, both of which are considered to be important factors in platelet aggregation and thrombus formation in vivo. These results in-

TABLE 1. EFFECTS OF PPAR γ AGONISTS IN REGARD TO PLATELET ACTIVATION AS ASSESSED IN IN VITRO STUDIES

Effect	Reference (year)
Platelet	
Reduction of TXA ₂	Akbayik et al. ²⁷ (2004) Hishinumu et al. ³⁰ (2000)
Reduction of sCD40L	Akbayik et al. ²⁷ (2004) Ray et al. ²⁸ (2006)
Macrophages/monocytes	
Reduction of MCP-1	Zeng et al. ³⁴ (2003)
Reduction of PAF	Sumita et al. ³¹ (2004)
Fibroblasts	
Reduction of collagen synthesis	Chen et al. ³⁵ (2004) Burgess et al. ³⁶ (2005)
Hepatic stellate cells	
Reduction of collagen synthesis	Galli et al. ³⁷ (2002)
Reduction of MCP-1	Marra et al. ³⁸ (2000)

dicates that pioglitazone administration is able to decrease platelet aggregation and delay intra-arterial thrombus formation in rats.⁴⁰ In another animal study, application of pioglitazone in an obese mouse model provided significant protection from thrombosis by prolonging the time to occlusive thrombosis by 40% and 68% at 7 and 15 weeks of age, respectively.³⁹ Also, following a diet challenge to promote diabetes in the same study, pioglitazone provided protection from occlusive thrombus formation, which was paralleled by significantly lower soluble P-selectin and platelet P-selectin expression, providing evidence of an antiplatelet effect.³⁹

In animal models, the amino acid L-arginine has an inhibitory effect on platelet activity.⁴⁴ L-Arginine is the natural substrate for NO synthesis,⁴⁵ activates fibrinolysis, and inhibits coagulation. L-Arginine administration exerts

beneficial effects in conditions associated with endothelial dysfunction and reduced NO synthesis. TZDs are capable of inhibiting the L-arginine degrading enzyme and of the NO-synthesis inhibitor asymmetric dimethylarginine (ADMA),⁴² thereby triggering a net increase of serum L-arginine.

Diabetes is associated with enhanced collagen-mediated platelet activation and an increase in extracellular matrix deposition.⁴⁶ TZDs decreased extracellular matrix deposition in a rat model of liver fibrosis and inhibited transforming growth factor β -induced collagen and fibrinogen synthesis in in vitro experiments.³⁷ PPAR γ ligands provide strong apoptotic signals to transformed T cells in concert with the anti-inflammatory mediator prostaglandin D₂.⁴⁷ In summary, a variety of TZD-induced antithrombotic actions have been reported from various animal models.

TABLE 2. EFFECTS OF PPAR γ AGONISTS ON PLATELET ACTIVATION AS ASSESSED IN ANIMAL STUDIES

Effect	Reference (year)
Mouse	
Prolongation of time to occlusive thrombus formation	Bodary et al. ³⁹ (2005)
Reduction of P-selectin	Bodary et al. ³⁹ (2005)
Rat	
Prolongation of time to occlusive thrombus formation	Li et al. ⁴⁰ (2005)
Increase in cNOS expression	Li et al. ⁴⁰ (2005)
Increase in thrombomodulin	Li et al. ⁴⁰ (2005)
Reduction of serum homocysteine	Murthy et al. ⁴¹ (2005)
Inhibition of ADMA	Wakino et al. ⁴² (2004)

RESULTS FROM CLINICAL TRIALS

Based on this comprehensive evidence of antithrombotic efficacy of TZDs *in vitro* and in animal experiments, it was speculated that PPAR γ activation may also modulate platelet activation and the release pattern of thrombocyte secretion products in patients with metabolic syndrome and type 2 diabetes. The investigation of the potentially associated clinical effects has just been started in recent years. It was initially shown with troglitazone that PPAR γ agonists decrease thrombogenicity by reducing PAI-1 and fibrinogen,^{48,49} and the same has meanwhile also been observed with pioglitazone and rosiglitazone. The comprehensive antithrombotic and anti-atherosclerotic efficacy was further demonstrated by reduction of several further biomarkers, including P-selectin, E-selectin, von Willebrand factor, monocyte chemoattractant protein 1 (MCP-1), matrix metalloproteinase (MMP)-9, and high sensitivity C-reactive protein (hsCRP) in numerous controlled clinical trials. An overview on these observations as well as the respective references is provided in Table 3.

The observed positive changes in these biomarkers of platelet function and vascular inflammation were accompanied by improvements in clinically relevant surrogate indicators of cardiovascular risk. In fact, TZDs are the first antidiabetic drug class that has been demonstrated to significantly treat atherosclerosis, which was indicated by a decreased intima-media thickness in several observations.⁶³⁻⁶⁶ This finding was independent from glycemic control⁶⁶ and translated into a significant reduction of macrovascular end points by pioglitazone versus placebo in the first appropriately performed large outcome study.^{67,68}

IMPLICATIONS OF THE ANTITHROMBOTIC EFFECTS OF TZDs

Insulin exerts antiplatelet activity by stimulating endothelial cell production of the platelet inhibitors prostacyclin and NO, an important mechanism mediating vasodilatation.⁶⁹ It was also suggested that pioglitazone administration decreases platelet aggregation and delays intra-arterial thrombus formation in rats, at

TABLE 3. CONFIRMED EFFECTS OF PPAR γ AGONISTS ON BIOMARKERS OF PLATELET FUNCTION AND VASCULAR INFLAMMATION AS DERIVED FROM CLINICAL TRIALS

Effect	Reference (year)
Reduction of PAI-1	Forseca et al. ⁴⁸ (1998) Kato et al. ⁴⁹ (2000) Kruszynska et al. ⁵⁰ (2000) Derosa et al. ⁵¹ (2005) Derosa et al. ⁵² (2005)
Reduction of P-selectin	Sidhu et al. ⁵³ (2004) Chu et al. ⁵⁴ (2005)
Reduction of E-selectin	Sidhu et al. ⁵³ (2003) Chu et al. ⁵⁴ (2005) Hetzel et al. ⁵⁶ (2005)
Reduction of von Willebrand factor	Sidhu et al. ⁵⁵ (2003)
Reduction of hsCRP	Haffner et al. ⁵⁷ (2002) Sidhu et al. ⁵⁵ (2003) Pfützner et al. ⁵⁸ (2005) Hetzel et al. ⁵⁶ (2005) Pfützner et al. ⁵⁹ (2006) Chu et al. ⁶⁰ (2006) Ghanim et al. ⁶¹ (2006)
Reduction of sCD40L	Marx et al. ⁶² (2003) Chu et al. ⁶⁰ (2006)
Reduction of MMP-9	Haffner et al. ⁵⁷ (2002) Marx et al. ⁶² (2003) Pfützner et al. ⁵⁸ (2005) Chu et al. ⁶⁰ (2006) Ghanim et al. ⁶¹ (2006)

least partially, by an increase in the expression of cNOS and thrombomodulin.⁴⁰ Under normal conditions, insulin action on platelet function leads to inhibition and dissolution of the newly formed thrombus in the circulation, also by directly suppressing the P2Y₁₂ pathway. The platelet P2Y₁₂ receptor plays a pivotal role in platelet aggregation, and its activation leads to thrombosis.⁷⁰ The P2Y₁₂ pathway is up-regulated in patients with type 2 diabetes in comparison to healthy subjects, leading to increased platelet aggregation and increased shear-induced platelet function, which appears to be independent of glycemic control and inflammatory status.^{24,71} It is of interest that insulin-treated patients with type 2 diabetes with longer disease duration show reduced effects of the P2Y₁₂ antagonist clopidogrel on ADP-induced platelet aggregation as compared with non-insulin-treated patients.⁷¹ The glucose-independent P2Y₁₂ up-regulation and the subsequent pro-inflammatory and prothrombotic state that is a characteristic of insulin-treated type 2 patients may be an explanation for the higher rates of restenosis or stent thrombosis

that has been observed in this patient population.⁷² Therefore, aggressive antithrombotic regimens, which may include novel platelet glycoprotein IIb/IIIa receptor inhibitors, novel P2Y₁₂ antagonists, and insulin sensitizers, are recommended for these patients.

Platelets have been identified as key mediators in inflammation. The prevention of release of pro-inflammatory molecules from platelets seems to be a direct effect of TZDs and may have significant implications for the treatment of the increased platelet aggregation and vascular inflammation in patients with diabetes.²⁸ While part of the antithrombotic activity of PPAR γ agonists can be attributed to direct action on the platelets, other TZD effects also indirectly contribute to the observed overall improvement of platelet function (Fig. 2).

TZDs reduce white blood cell count and P-selectin-positive platelets and acute-phase proteins like C-reactive protein, amyloid A, and fibrinogen.^{58,73} In addition, TZDs improve endothelial cell function in animals and humans. Pioglitazone administration in streptozotocin-diabetic rats lowered blood pressure, protected

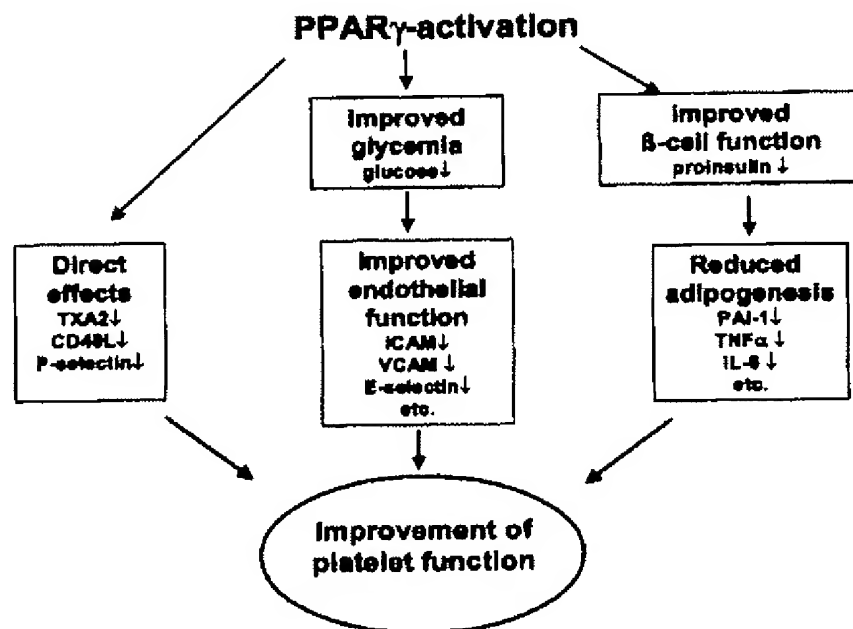


FIG. 2. Factors contributing to the improvement in platelet function by TZD therapy. ICAM, intercellular adhesion molecule; TNF α , tumor necrosis factor- α ; VCAM, vascular cell adhesion molecule.

against oxidative stress, and restored endothelial function, which prevented the breakdown of NO and increased NO levels.⁷⁴

High homocysteine levels correlate with progression of vascular disease because of elevation of adverse effects of platelets.³⁴ Homocysteine up-regulates MCP-1 secretion in monocytes; however, this effect can be prevented by PPAR γ activation.⁷⁵ The potency of TZD treatment to reduce serum homocysteine levels is controversial: in a rat model, rosiglitazone reduced serum homocysteine levels,⁴¹ whereas this effect was not observed in clinical trials.⁷⁶

In the process of β -cell dysfunction development in type 2 diabetes and metabolic syndrome, exhaustion of the pro-insulin cleavage enzymes in the β -cell leads to increasing secretion of intact pro-insulin, which has been demonstrated to be an independent cardiovascular risk factor.⁷⁷ Pro-insulin stimulates adipogenesis resulting in increased secretion of pro-inflammatory markers, such as IL-6, tumor necrosis factor- α , and PAI-1. PAI-1 is the primary inhibitor of endogenous-type fibrinolysis, and its release leads to increased risk of cardiovascular events.^{78,79} Treatment of patients with type 2 diabetes with rosiglitazone or pioglitazone has been demonstrated to reduce the levels of circulating intact pro-insulin independent from an improvement of glycemic control, and in parallel with improvement of several other cardiovascular risk factors.^{58,59,66} Further supporting effects are the reduction in cytokine expression by several cell types⁸⁰ and a decrease in MMP production.^{81,82} Elevated MMP-9 and the presence of the described rupture-prone plaques of high lipid composition increase the risk of thrombotic events. In combination with dysfunctional hyperactive and hyperaggregatory platelets of insulin-resistant patients with diabetes, released cell detritus from a ruptured plaque into circulation may well cause ischemic events with fatal outcome. This overall risk of ischemic events can be reduced by TZDs, which has recently been demonstrated with a reduction of stroke, myocardial infarction, and death by 16% over 3 years in the first large cardiovascular outcome trial with pioglitazone, the PROactive Study.⁶⁷

The use of TZDs, exerting their effects through activation of PPAR γ as the therapeutic means for glycemic control and anti-inflammatory and antithrombotic effects, represents an important adjunctive approach in the required and necessarily more aggressive antithrombotic therapy in patients with type 2 diabetes mellitus.

REFERENCES

1. Ross R: Atherosclerosis: an inflammatory disease. *N Engl J Med* 1999;340:115-126.
2. Ferroni P, Basili S, Davi G: Platelet activation, inflammatory mediators and hypercholesterolemia. *Curr Vasc Pharmacol* 2003;1:157-169.
3. Viles-Gonzales JF, Anand SX, Valdiviezo C, Zafar U, Hunter R, Sanz J, Rius T, Poon M, Fuster V, Badimon JJ: Update in atherothrombotic disease. *Mount Sinai J Med* 2004;71:197-208.
4. Haffner SM, Lehto S, Ronnema T, Pyorala K, Laakso M: Mortality from coronary heart disease in subjects with type 2 diabetes and in nondiabetic subjects with and without prior myocardial infarction. *N Engl J Med* 1998;339:229-234.
5. Lüscher TF, Creager MA, Beckman JA, Cosentino F: Diabetes and vascular disease: pathophysiology, clinical consequences, and medical therapy: part II. *Circulation* 2003;108:1655-1661.
6. Yngen M, Norhammar A, Hjelm Dahl P, Wallen NH: Effects of improved metabolic control on platelet reactivity in patients with type 2 diabetes mellitus following coronary angioplasty. *Diabetes Vasc Dis Res* 2006;3:52-56.
7. Dandona P, Ajlada A, Chaudhuri A, Mohanty P, Garg R: Metabolic syndrome: a comprehensive perspective based on interactions between obesity, diabetes, and inflammation. *Circulation* 2005;111:1448-1454.
8. Malek AM, Alper SL, Izumo S: Hemodynamic shear stress and its role in atherosclerosis. *JAMA* 1999;282:2035-2042.
9. Kintzsch U, Marx N, Koenig W, Pfützner A, Forst T, Schnell O: Cardiodiabetology: the present state, epidemiology, pathophysiology, therapy, clinic and practice [in German]. *Diabetes Stoffw Herz* 2006;15:33-37.
10. Ferroni P, Basili S, Falco A, Davi G: Platelet activation in type 2 diabetes mellitus. *J Thromb Haemost* 2004;2:1282-1291.
11. Stratmann B, Tschöpe D: Pathobiology and cell interactions of platelets in diabetes. *Diabetes Vasc Dis Res* 2005;2:16-23.
12. Vaidya VR, Boden G, Rao AK: Platelet and monocyte activation by hyperglycemia and hyperinsulinemia in healthy subjects. *Platelets* 2006;17:577-585.
13. Watala C, Boncler M, Gresner P: Blood platelet abnormalities and pharmacological modulation of

- platelet reactivity in patients with diabetes mellitus. *Pharmacol Rep* 2005;57(Suppl):42-58.
14. Colwell JA, Nesto RW: The platelet in diabetes: focus on prevention of ischemic events. *Diabetes Care* 2003;26:2181-2188.
 15. Varughese GI, Patel JV, Tomson J, Lip GY: The prothrombotic risk of diabetes mellitus in atrial fibrillation and heart failure. *J Thromb Haemost* 2005;3:2811-2813.
 16. Creager MA, Lüscher TF, Cosentino F, Beckman JA: Diabetes and vascular disease: pathophysiology, clinical consequences, and medical therapy: part I. *Circulation* 2003;108:1527-1532.
 17. Denys MM, Tolley ND, Bunting M, Schwertz H, Jiang H, Lindemann S, Yost CC, Rubner FJ, Albertine KH, Swoboda KJ, Fratto CM, Tolley E, Kraiss LW, McIntyre TM, Zimmerman GA, Weyrich AS: Escaping the nuclear confines: signal-dependent pre-mRNA splicing in anuclear platelets. *Cell* 2005;122:379-391.
 18. Henn V, Slupsky JR, Grafe M, Anagnostopoulos I, Forster R, Müller-Berghaus G, Kroczeck RA: CD40 ligand on activated platelets triggers an inflammatory reaction of endothelial cells. *Nature* 1998;391:591-594.
 19. Davi G, Ferroni P, Basili S: Platelet activation, inflammatory mediators and hypercholesterolemia. *Curr Vasc Pharmacol* 2003;1:157-169.
 20. Shoji T, Koyama H, Fukumoto S, Maeno T, Yokoyama H, Shinohara K, Emoto M, Shoji T, Inaba M, Nishizawa Y: Platelet-monocyte aggregates are independently associated with occurrence of carotid plaques in type 2 diabetic patients. *J Artheroscler Thromb* 2005;12:344-352.
 21. Levi M, van der Poll T, Büller H: Bidirectional relation between inflammation and coagulation. *Circulation* 2004;109:2698-2704.
 22. Chakraborty K, Sinha AK: The role of insulin as a humoral antithrombotic factor. *Bioassays* 2004;26:91-98.
 23. Tamminen M, Lassila R, Westerbacka J, Vehkavaara S, Yki-Jarvinen H: Obesity is associated with impaired platelet-inhibitory effect of acetylsalicylic acid in non-diabetic subjects. *Int J Obes Relat Metab Disord* 2003;27:907-911.
 24. Ferreira IA, Mocking AI, Feijge MA, Corter G, van Haften TW, Heemskerk JW, Akkerman JW: Platelet inhibition by insulin is absent in type 2 diabetes mellitus. *Arterioscler Thromb Vasc Biol* 2006;26:417-422.
 25. Lim HS, Blann AD, Lip GY: Soluble CD40 ligand, soluble P-selectin, interleukin-6, and tissue factor in diabetes mellitus: relationships to cardiovascular disease and risk factor intervention. *Circulation* 2004;109:2524-2528.
 26. Vishnevetsky D, Kiyani VA, Gandhi PJ: CD40 ligand: a novel target in the fight against cardiovascular disease. *Ann Pharmacother* 2004;38:1500-1508.
 27. Akbiyik F, Ray DM, Gettings KF, Blumberg N, Francis CW, Phipps RP: Human bone marrow megakaryocytes and platelets express PPAR γ , and PPAR γ agonists blunt platelet release of CD40 ligand and thromboxanes. *Blood* 2004;104:1361-1368.
 28. Ray DM, Spinelli SL, O'Brien JJ, Blumberg N, Phipps RP: Platelets as a novel target for PPAR γ ligands: implications for inflammation, diabetes, and cardiovascular disease. *BioDrugs* 2006;20:231-241.
 29. Jiang DQ, Chu LX, Liu ZY, Zhang SB: Pioglitazone decreased CD40/CD40L expression on human umbilical vein endothelial cells induced by oxidized low-density lipoprotein. *Clin Chim Acta* 2006;370:94-99.
 30. Hishinuma T, Yamazaki T, Mizugaki M: Troglitazone has a reducing effect on thromboxane production. *Prostaglandins Other Lipid Mediat* 2000;62:135-143.
 31. Sumita C, Maeda M, Fujio Y, Kim J, Fujitsu J, Kasayama S, Yamamoto I, Azuma J: Pioglitazone induces plasma platelet activating factor-acetylhydrolase and inhibits platelet-activating factor-mediated cytoskeletal reorganization in macrophage. *Biochim Biophys Acta* 2004;1673:115-121.
 32. Benson S, Wu J, Padmanabhan S, Kurtz TW, Pershadsingh HA: Peroxisome proliferator-activated receptor (PPAR)-gamma expression in human vascular smooth muscle cells: inhibition of growth, migration, and c-fos expression by the peroxisome proliferator-activated receptor (PPAR)-gamma activator troglitazone. *Am J Hypertens* 2000;13:74-82.
 33. DeDios ST, Bruemner D, Dilley RJ, Ivey ME, Jennings GLR, Law RE, Little PJ: Inhibitory activity of clinical thiazolidinediones peroxisome proliferator activating receptor γ ligands toward internal mammary artery, radial artery, and saphenous vein smooth muscle cell proliferation. *Circulation* 2003;107:2548-2550.
 34. Zeng X, Dai J, Remick DG, Wang X: Homocysteine mediated expression and secretion of monocyte chemoattractant protein-1 and interleukin-8 in human monocytes. *Circ Res* 2003;93:311-320.
 35. Chen K, Chen J, Li D, Zhang X, Mehta JL: Angiotensin II regulation of collagen type I expression in cardiac fibroblasts: modulation by PPAR-gamma ligand pioglitazone. *Hypertension* 2004;44:655-661.
 36. Burgess HA, Daugherty LE, Thatcher TH, Lakatos HF, Ray DM, Redonnet M, Phipps RP, Sime PJ: PPAR γ agonists inhibit TGF-beta induced pulmonary myofibroblasts differentiation and collagen production: implications for therapy of lung fibrosis. *Am J Physiol Lung Cell Mol Physiol* 2005;288:L1146-L1153.
 37. Galli A, Crabb DW, Ceri E, Salzano R, Mello T, Svegliati-Baroni G, Ridolfi F, Trozzi L, Surrenti C, Casini A: Antidiabetic thiazolidinediones inhibit collagen synthesis and hepatic stellate cell activation in vivo and in vitro. *Gastroenterology* 2002;122:1924-1940.
 38. Marra F, Efsen E, Romanelli RG, Caligiuri A, Pastacaldi S, Batignani G, Bonacci A, Caporale R, Laffi G, Pinzani M, Gentilini P: Ligands of peroxisome proliferator-activated receptor gamma modulate profibrogenic and proinflammatory actions in hepatic stellate cells. *Gastroenterology* 2000;119:466-478.
 39. Bodary PF, Vargas FB, King SAD, Jongeward KL, Wickenheiser KJ, Eitzman DT: Pioglitazone protects against thrombosis in a mouse model of obesity and

- insulin resistance. *J Thromb Haemost* 2005;3:2149-2153.
40. Li D, Chen K, Sinha N, Zhang X, Wang Y, Sinha AK, Romeo F, Mehta JL: The effects of PPAR-gamma ligand pioglitazone on platelet aggregation and arterial thrombus formation. *Cardiovasc Res* 2005;65:907-912.
 41. Murthy SN, Obregon DF, Chattergon NN, Fonseca NA, Mondal D, Dunne JB, Diez JC, Jeter JR Jr, Kadowitz PJ, Agrawal KC, Mc Namara DB, Fonseca VA: Rosiglitazone reduces serum homocysteine levels, smooth muscle proliferation, and intimal hyperplasia in Sprague-Dawley rats fed a high methionine diet. *Metabolism* 2005;54:645-652.
 42. Wakino S, Hayashi K, Kanda T, Tatematsu S, Homma K, Yoshioka K, Takamatsu I, Saruta T: Peroxisome proliferator-activated receptor γ ligands inhibit Rho/Rho kinase pathway by inducing protein tyrosine phosphatase SHP-2. *Circ Res* 2004;95:e45-e55.
 43. Kasono K, Nishida J, Tamemoto H, Fudaka K, Nami K, Kajio H, Masatoshi K, Kanazawa Y, Kawakami M: Thiazolidinediones increase the number of platelets in immune thrombocytopenic purpura mice via inhibition of phagocytic activity of the reticulo-endothelial system. *Life Sci* 2002;71:2037-2052.
 44. Leoncini G, Pruzzese D, Signorello MG: The L-arginine/NO pathway in the early phases of platelet stimulation by collagen. *Biochem Pharmacol* 2005;69:289-296.
 45. Cylwik D, Mogielnicki A, Buczek W: L-Arginine and cardiovascular system. *Pharmacol Rep* 2005;57:14-22.
 46. Cabeza N, Li Z, Schulz C, Kremmer E, Massberg S, Bültmann A, Gawaz M: Surface expression of collagen receptor α_2 receptor- γ /glycoprotein VI is enhanced on platelets in type 2 diabetes and mediates release of CD40 ligand and activation of endothelial cells. *Diabetes* 2004;53:2117-2121.
 47. Harris SC, Phipps RP: Prostaglandin D₂, its metabolic 15-d- PGJ_2 , and peroxisome proliferator activated receptor-gamma agonists induce apoptosis in transformed, but not normal, human T lineage cells. *Immunology* 2002;105:23-34.
 48. Fonseca VA, Reynolds T, Hemphill D, Randolph C, Wall J, Valiquet TR, Graveline J, Fink LM: Effect of troglitazone on fibrinolysis and activated coagulation in patients with non-insulin-dependent diabetes mellitus. *J Diabetes Complications* 1998;12:181-186.
 49. Kato K, Yamada D, Midorikawa S, Sato W, Watanabe T: Improvement by the insulin-sensitizing agent, troglitazone, of abnormal fibrinolysis in type 2 diabetes mellitus. *Metabolism* 2000;49:662-665.
 50. Kruszynska YT, Yu JG, Olefsky JM, Sobel BE: Effects of troglitazone on blood concentrations of plasminogen activator inhibitor 1 in patients with type 2 diabetes and in lean and obese normal subjects. *Diabetes* 2000;49:633-669.
 51. Derosa G, Cicero AF, Gaddi A, Ragonesi PD, Piccinini MN, Fogari E, Salvadeo S, Ciccarelli L, Fogari R: A comparison of the effects of pioglitazone and rosiglitazone combined with glimepiride on prothrombotic state in type 2 diabetic patients with the metabolic syndrome. *Diabetes Res Clin Pract* 2005;69:5-13.
 52. Derosa G, Gaddi AV, Piccinini MN, Ciccarelli L, Salvadeo S, Peros E, Ghelfi M, Ferrari J, Cicero AF: Antithrombotic effects of rosiglitazone-metformin versus glimepiride-metformin combination therapy in patients with type 2 diabetes mellitus and metabolic syndrome. *Pharmacotherapy* 2005;25:637-645.
 53. Sidhu JS, Cowan D, Tooze JA, Kaski JC: Peroxisome proliferator-activated receptor-gamma agonist rosiglitazone reduces circulating platelet activity in patients without diabetes mellitus who have coronary artery disease. *Am Heart J* 2004;147:e25.
 54. Chu JW, Abbasi F, Lamendola C, McLaughlin T, Reaven GM, Tsao PS: Effect of rosiglitazone treatment on circulating vascular and inflammatory markers in insulin-resistant subjects. *Diabetes Vasc Dis Res* 2005;2:37-41.
 55. Sidhu JS, Cowan D, Kaski JC: The effects of rosiglitazone, a peroxisome proliferator-activated receptor-gamma agonist, on markers of endothelial cell activation, C-reactive protein, and fibrinogen levels in non-diabetic coronary artery disease patients. *J Am Coll Cardiol* 2003;42:1757-1763.
 56. Hetzel J, Balletshofer B, Rittig K, Walcher D, Kratzer W, Hombach V, Häring HU, Koenig W, Marx N: Rapid effects of rosiglitazone treatment on endothelial function and inflammatory biomarkers. *Arterioscler Thromb Vasc Biol* 2005;25:1804-1809.
 57. Haffner SM, Greenberg AS, Weston WM, Chen H, Williams K, Freed MI: Effect of rosiglitazone treatment on nontraditional markers of cardiovascular disease in patients with type 2 diabetes mellitus. *Circulation* 2002;106:679-684.
 58. Pfützner A, Marx N, Lübken G, Langenfeld M, Walcher D, Konrad T, Forst T: Improvement of cardiovascular risk markers by pioglitazone is independent from glycemic control. *J Am Coll Cardiol* 2005;45:1925-1931.
 59. Pfützner A, Schöndorf T, Seidel D, Winkler K, Matthaei S, Hamann A, Forst T: Impact of rosiglitazone on β -cell function, insulin resistance and adiponectin concentrations—results from a double blind oral combination study with glimepiride. *Metabolism* 2006;55:20-25.
 60. Chu CS, Lee KT, Lee MY, Su HM, Voon WC, Sheu SH, Lai WT: Effects of rosiglitazone alone and in combination with atorvastatin on nontraditional markers of cardiovascular disease in patients with type 2 diabetes mellitus. *Am J Cardiol* 2006;97:646-650.
 61. Ghanim H, Dhindsa S, Aljada A, Chaudhuri A, Viswanathan P, Dandona P: Low dose rosiglitazone exerts an anti-inflammatory effect with an increase in adiponectin independently of free fatty acid (FFA) fall and insulin sensitization in obese type 2 diabetics. *J Clin Endocrinol Metab* 2006;91:3553-3558.
 62. Marx N, Imhof A, Froehlich J, Siam L, Itner J, Wierse G, Schmidt A, Maerz W, Hombach V, Koenig W: Effect of rosiglitazone treatment on soluble CD40L in

- patients with type 2 diabetes and coronary artery disease. *Circulation* 2003;107:1954-1957.
63. Koshiyama H, Shimono D, Kuwamura N, Minamikawa J, Nakamura Y: Inhibitory effect of pioglitazone on carotid arterial wall thickness in type 2 diabetes. *J Clin Endocrinol Metab* 2001;86:3452-3456.
 64. Mazzone T, Meyer PM, Feinstein SB, Davidson MH, Kondos GT, D'Agostino RB, Perez A, Provost JC, Haffner SM: Effect of pioglitazone compared with glimepiride on carotid intima-media thickness in type 2 diabetes. A randomized trial. *JAMA* 2006;295:2572-2581.
 65. Hedblad B, Zambanini A, Nilsson P, Janzon L, Berglund G: Rosiglitazone and carotid IMT progression rate in a mixed cohort of patients with type 2 diabetes and the insulin resistance syndrome: main results from the Rosiglitazone Atherosclerosis Study. *J Intern Med* 2007;261:293-3057.
 66. Langenfeld M, Forst T, Hohberg C, Kann P, Lübken G, Konrad T, Füllert S, Sachara C, Pfützner A: Pioglitazone decreases carotid intima-media thickness independent of glycemic control in patients with type 2 diabetes mellitus. *Circulation* 2005;111:2525-2531.
 67. Dormandy JA, Charbonnel B, Eckland EJA, Erdmann E, Massi-Benedetti M, Moules IK, Skene AM, Tan MH, Lefebvre PJ, Murray GD, Standl E, Wilcox RG, Wilhelmssen L, Betteridge J, Birkeland K, Colay A, Heine RJ, Koranyi L, Laakso M, Mokan M, Norkus A, Pirags V, Podar T, Scheen A, Scherbaum W, Schemthaler G, Schmitz O, Skirha J, Smith U, Taton J: PROactive Investigators: Secondary prevention of macrovascular events in patients with type 2 diabetes in the PROactive Study (PROspective pioglitazone Clinical Trial in macroVascular Events): a randomised controlled trial. *Lancet* 2005;366:1279-1289.
 68. Wilcox R, Bousser MC, Betteridge DJ, Schemthaler G, Pirags V, Kupfer S, Dormandy J: PROactive Investigators: Effects of pioglitazone in patients with type 2 diabetes with or without previous stroke: results from PROactive (PROspective pioglitazone Clinical Trial in macroVascular Events). *Stroke* 2007;38:865-873.
 69. Zeng G, Quon MJ: Insulin-stimulated production of nitric oxide is inhibited by wortmannin: direct measurement in vascular endothelial cells. *J Clin Invest* 1996;98:894-898.
 70. Hechler B, Cattaneo M, Gachet C: The P2 receptors in platelet function. *Semin Thromb Hemost* 2005;31:150-161.
 71. Angiolillo DJ, Bernado E, Ramirez C, Costa MA, Sabate M, Jimenez-Quevedo P, Hernandez R, Moreno R, Escaned J, Alfonso F, Banuelos C, Bass TA, Macaca C, Fernandez-Ortiz A: Insulin therapy is associated with platelet dysfunction in patients with type 2 diabetes mellitus on dual oral antiplatelet treatment. *J Am Coll Cardiol* 2006;48:298-304.
 72. Guagliumi G, Musumeci G: Revascularization of diabetic patients: are drug-eluting stents the solution? *Ital Heart J* 2005;6:507-513.
 73. Buckingham RE: Thiazolidinediones: pleiotropic drugs with potent anti-inflammatory properties for tissue protection. *Hepatol Res* 2005;33:167-170.
 74. Majithiya JB, Paramar AN, Balaraman R: Pioglitazone, a PPARgamma agonist, restores endothelial function in aorta of streptozotocin-induced diabetic rats. *Cardiovasc Res* 2005;66:150-161.
 75. Flisman EZ, Tenenbaum A, Motro M, Adler Y: Oral antidiabetic therapy in patients with heart disease. A cardiologic standpoint. *Herz* 2004;29:290-298.
 76. Sarafidis PA, Lasaridis AN, Nilsson PM, Mouslech TF, Hitoglou-Mekedou AD, Stafylas PC, Kazakos KA, Yovos JC, Tourkantonis AA: The effects of rosiglitazone on novel atherosclerotic risk factors in patients with type 2 diabetes mellitus and hypertension. An open-label observational study. *Metabolism* 2005;54:1236-1242.
 77. Pfützner A, Kann PH, Pfützner AH, Kunt T, Larbig M, Weber MM, Forst T: Intact and total proinsulin: new aspects for diagnosis and treatment of type 2 diabetes mellitus and insulin resistance. *Clin Lab* 2004;50:567-573.
 78. Schneider DJ, Nordt TK, Sobel BE: Stimulation by proinsulin of expression of plasminogen activator inhibitor type-I in endothelial cells. *Diabetes* 1992;41:890-895.
 79. Nordt TK, Bode C, Sobel BE: Stimulation in vivo of expression of intra-abdominal adipose tissue plasminogen activator inhibitor Type I by proinsulin. *Diabetologia* 2001;44:1121-1124.
 80. Jlang C, Ting AT, Seed B: PPARgamma agonists inhibit production of monocyte inflammatory cytokines. *Nature* 1998;391:82-86.
 81. Marx N, Schönbeck U, Lazar MA, Libby P, Plutzky J: Peroxisome proliferator-activated receptor gamma activators inhibit gene expression and migration in human vascular smooth muscle cells. *Circ Res* 1998;83:1097-1103.
 82. Marx N, Froehlich J, Siam L, Ittner J, Wierse G, Schmidt A, Schramagl H, Hombach V, Koenig W: Antidiabetic PPAR gamma-activator rosiglitazone reduces MMP-9 serum levels in type 2 diabetic patients with coronary artery disease. *Arterioscler Thromb Vasc Biol* 2003;23:283-288.

Address reprint requests to:
 Dr. Thomas Schöndorf, Ph.D.
 Associate Professor
 ikfe GmbH
 Ernst-Ludwig-Straße 6-8
 D-55116 Mainz, Germany
 E-mail: thomass@ikfe.de